SKIN PEPTIDE DEFENCES OF AFRICAN CLAWED FROGS (*Xenopus laevis*) AND NEW ZEALAND *Litoria* FROGS AGAINST BACTERIAL DERMATOSEPTICEMIA

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by Ermin Schadich

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Abstract

In frogs, part of the important immune defence system of their skin is the secretion of antimicrobial peptides from granular glands. This study investigated the immune function of skin peptides in protection against bacterial pathogens associated with infectious bacterial dermatosepticemia under a number of environmental conditions and at certain stages of the life cycle of frogs. The natural peptide mixture of skin peptides was collected from skin secretions of three semi-aquatic *Litoria* frog species *L. aurea*, *L. raniformis* and *L. ewingii* and aquatic *Xenopus laevis* and assayed for activity against the bacterial pathogens: *Aeromonas hydrophila*, *Chryseobacterium meningosepticum*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Serratia liquefaciens*. The peptide mixtures of three frog species *Xenopus laevis*, *Litoria aurea* and *Litoria raniformis* showed activity against *C. freundii*, *C. meningosepticum*, *K. pneumoniae* and *P. aeruginosa in vitro* indicating a likely protective function. One *Litoria* species, *L. ewingii*, had a peptide mixture that did not have activity against any pathogen. Subsequently, in experimental exposure of animals to the pathogen *K. pneumoniae*, this species was found to be susceptible to disease while the other sympatric species *L. raniformis* was found to be resistant. A strong correlation was shown between composition of skin peptides and resistance to disease.

A comparison of the production and activity of skin peptides from four frog species showed the aquatic *X. laevis* to have more effective immune defence against bacterial pathogens than three tested *Litoria* species. *X. laevis* produced significantly greater amount of bioactive peptide mixture than three tested *Litoria* species.

Three pathogens *A. hydrophila*, *P. mirabilis* and *S. liquefaciens* are abundant components of the skin microbiota of healthy frogs and were found to be resistant to the peptide mixtures of all four frog species tested. It was shown that one pathogen, *A. hydrophila*, had the ability to secrete proteases which could inactivate skin peptides. Thus while skin peptides could function against several pathogens, some pathogens might have co-evolved to resist skin peptides.

A comparison of the peptide mixtures from skin secretions of adults, metamorphs and larvae of *L. ewingii* using liquid chromatography-mass spectrometry analyses showed that peptide mixtures of post metamorphic animals, adults and metamorphs, had a species-specific profile that included the antimicrobial peptide uperin 7.1, while the larval peptide mixture did not contain uperin 7.1 or any other known species-specific peptide. This finding indicates the absence of a secretory mechanism that could compensate for the absence of granular glands in larvae.

Analyses of the production and activity of skin peptides of *L. raniformis* after exposure to two different environmental stressors, low environmental temperature and pesticide carbaryl, showed that the total amount of bioactive peptide was significantly reduced which could consequently increase susceptibility to disease. Thus suppression of skin peptides could be a possible mechanism for synergism between the important stressors and pathogens in disease development.

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Chapter 1: Introduction

1.1 The Significance of Research on the Immune System of Frogs

Research on the immune system of frogs and other amphibians is an area of growing scientific interest. The interest is due to the association between infectious disease and declines of amphibian populations, which have occurred in both pristine and compromised habitats on all six continents (Carey, *et al.*, 1999, Daszak *et al*., 1999). While chytridiomycosis, a fungal disease of skin caused by the chytrid fungus *Batrachochytrium dendrobatidis*, has received much attention in Australia and Central America (Berger *et al*., 1998), other viral and bacterial diseases have been observed. In Great Britain, disease caused by *Ranavirus* (*Iridoviridae*) has been associated with mortality of the Common Frog (*Rana temporaria*) (Cunningham *et al*., 1996). The opportunistic bacterium *Aeromonas hydrophila* is associated with bacterial dermatosepticemia, a systematic bacterial disease that caused local population die-offs of two *Rana* species, *R. muscosa* and *R. sylvatica* (Nyman, 1986, Bradford, 1991). Other bacteria associated with mortality, especially among captive populations, are different Gram-negative species including *Chryseobacterium indologenes*, *Chryseobacterium meningosepticum*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aerugionsa* and *Serratia liquefaciens* (Glorioso *et al*., 1974, Anver and Pond, 1984, Olson *et al*., 1992, Taylor *et al*., 2001).

Emergence of these diseases in wild populations of frogs was surprising as all of the pathogens associated with disease are also found in healthy animals indicating their immune systems are sufficiently protective against these pathogens to establish a co-evolutionary

relationship with them (Hird *et al*., 1981, Pearson, 1998, Cullen and Owens, 2002, Schlaepfer *et al*., 2007). The wide range of pathogens and their relationship with their hosts suggests that disease itself cannot exclusively explain global declines of frog populations and other factors need to be considered. Stressors associated with decline of frogs in areas affected by disease, such as climate change and air-born pollutants, could suppress the immune systems of wild frogs and increase susceptibility to disease (Drost and Fellers, 1996, Blaustein *et al*., 1998, Beecher, 2006, Alford *et al*., 2007, Davidson and Knapp, 2007). Research on the functions of frog immune systems against invading pathogens and the effects of stressors on these immune systems should lead to a better understanding of the mechanisms of disease development and subsequent decline of frog populations.

1.2 The Immune System of Frogs

Frogs have the full complement of immune systems as higher vertebrates including both adaptive and innate immunity (Du Pasquier *et al*., 1989, Carey *et al*., 1999, Woodhams *et al*., 2007). Innate immunity involves different mechanisms that can provide an instant response nonspecifically to many different pathogens. In frogs, the innate immune defences include antimicrobial peptides (Simmaco *et al*., 1998, Rollins-Smith *et al*., 2005), a complement lytic system (Green and Cohen, 1977), phagocytic macrophages, granulocytes (Manning and Horton, 1982) and natural killer cells (NK cells) (Horton *et al*., 1996). As in other vertebrates, the adaptive immunity of frogs responds to previously encountered pathogens by recognition of their antigens. Its cellular components include different kinds of lymphocytes while its extracellular component (humoral component) includes specialized immunoglobulin proteins (antibodies) which are produced in specialized lymphocytes (B cells) (Du Pasquier *et al*.,

1989). It also includes two different protein complexes called major histocompatibility complex (MHC) I and II for presentation of endogenously and externally derived antigens respectively (Du Pasquier *et al*., 1989). The MHC I proteins are expressed on the membrane of every single cell of the bodies of adult frogs while the MHC II proteins are expressed on the limited number of T and B cells and the specialized macrophages called “antigen presenting cells” (Du Pasquier and Flajnik, 1990). Although frogs do not have lymph nodes and bone marrow, they do have a thymus and a spleen, which serve as central and peripheral lymphoid organs respectively. Therefore, frogs possess an immune system similar to higher vertebrates, including humans and other mammals.

Although the adaptive immune system of frogs has all components of the mammalian adaptive immune system, its responses to invading pathogens are too slow to evolve to provide sufficient protection in skin of poikilothermic animals like frogs whose physiological processes depend on the environmental temperature (Simmaco *et al*., 1998, Carey *et al*., 1999). Similarly, humoral innate immunity, including complement mediated effects, cannot provide protection from invading bacteria in frogs (Green and Cohen, 1977, Simmaco *et al*., 1998). Therefore, the specialised innate immune defences of skin such as the antimicrobial peptides might be essential in protection from invading pathogens.

1.3 Skin Antimicrobial Peptides

Antimicrobial peptides are found in microorganisms, plants, insect *haemolymph*, and the lungs, gastrointestinal tract and intestinal cells of mammals, as well as in frog skin secretions (Bevins and Zasloff, 1990, Barra and Simmaco, 1995, Rinaldi, 2002).

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Frogs have two types of skin glands that are located in the dermis: granular and mucous glands (Fig. 1) (Duellman and Trueb, 1986, Stebbins and Cohen, 1995, Shepherd *et al*., 1998). The function of mucous glands is to secrete mucus to keep the skin continuously moist for efficient respiration and thermoregulation (Duellman and Trueb, 1986, Stebbins and Cohen, 1995). Mucous and granular glands may occur in aggregations, or as macroglands visible as raised areas of skin described variously as parotid glands or dorsolateral folds of some species (Duellman and Trueb, 1986, Tyler, 1987). Many frog species lack these aggregations, and in these species glands are distributed evenly throughout the integument (Duellman and Trueb, 1986, Steinborner *et al*., 1997).

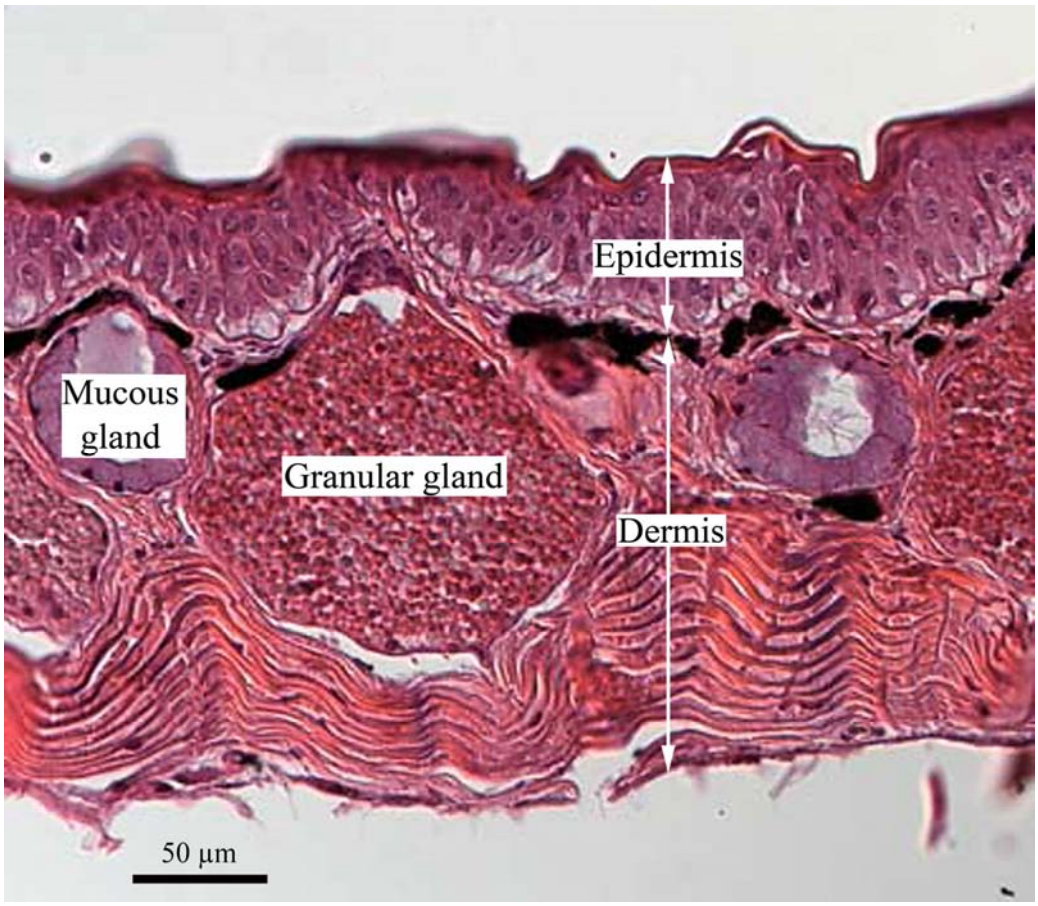

Fig. 1. Skin glands of adult Australian Southern Bell Frog (*Litoria raniformis*).

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Antimicrobial peptides are synthesized in granular glands (Duellman and Trueb, 1986, Erspamer, 1994, Shepherd *et al*., 1998) and secreted onto the skin surface in response to stimuli of sympathetic nervous system such as electric shock, administration of norepinephrine, injury, predation, handling or other stressors (Tyler *et al*., 1992, Rollins-Smith *et al*., 2002, Woodhams, 2003). As skin secretions have many possible functions such as defence against predators, mediation of pain and wound healing, in addition to antimicrobial peptides, the secretory epithelial cells also synthesize a variety of other peptide and non-peptide compounds that may function as toxins, hormones and neurotransmitters (Bevins and Zasloff, 1990, Barthalmus, 1994, Erspamer, 1994, Doyle *et al*., 2002). All of these compounds are stored as deposits in the lumen of granular glands and secreted together with antimicrobial peptides by the mechanism of holocrine secretions (Erspamer, 1994). After depletion, the deposits of skin peptides may be restored within 6 days to 2 weeks (Erspamer, 1994, Rollins-Smith *et al*., 2005).

Skin peptides, identified by their ability to inhibit microbial growth, have been isolated from skin secretions of frog species belonging to 11 different genera including *Ascaphus*, *Bombina*, *Bufo*, *Crinia*, *Hyla*, *Leptodactylus*, *Litoria*, *Phyllomedusa*, *Rana*, *Uperoleia* and *Xenopus* (reviewed in Pukala *at el*. 2006). In general, they are small peptides (11-40 amino acid residues) that have a positive net charge (reviewed in Pukala *at el*. 2006). They function by binding the negatively charged components of the membranes of different microbial cells and forming α-helical structures which cause perturbation of membranes and cell lysis (Zasloff, 1987, Matsuzaki *et al*., 1995, Shai, 2002). They have been shown to have activity both against frog invading pathogens *B. dendrobatidis* and *Ranavirus* and different human bacterial pathogens (Zasloff, 1987, Chinchar, *et al*., 2001, Rollins-Smith *et al*., 2002, Chinchar, *et al*.,

2004, Woodhams *et al*., 2006, Woodhams *et al*., 2007). To date, however, only peptides of the African Clawed frog (*Xenopus laevis*), including magainin 2, and peptides with amino-terminal glycine (PGL^a), have been tested for activity against one bacterium pathogenic to frogs, *A. hydrophila* (Rollins-Smith *et al*., 2002). The activity of these peptides against other bacterial pathogens, and activities of other skin peptides from all other species, have not previously been investigated.

Frog skin secretions contain a mixture of different peptides that have activity against invading pathogens (Simmaco *et al*., 1998, Rollins-Smith *et al*., 2002, Rollins-Smith *et al* 2005, Woodhams *et al*., 2006, Woodhams *et al*., 2007). The action of the peptide mixture is thought to be more important in protection against bacterial pathogens than the action of any single peptide component, with possible synergistic effects occurring (Westerhoff *et al*., 1995, Rollins-Smith *et al*., 2002). As each frog species secretes its own unique set of peptides, significant variation occurs among species in the number, structure and antimicrobial activity of these peptides (Vanhoye *et al*., 2003, Conlon *et al*., 2004).

On skin surface, the regulatory mechanism of activation of skin peptides involves proteases. The peptides are synthesized as large precursor peptides; after synthesis, a signal sequence is removed from the precursor peptides by an endoprotease and the smaller bio-inactive peptide is transported and stored in the granular glands. The peptide is activated by another protease when released from granular glands. In order to prevent self-injury, a third endoprotease deactivates the peptides within 15-30 minutes (Resnick, *et al*., 1991, Steinborner *et al*., 1997, Bowie *et al*. 1999). It is possible that some bacteria that are resistant to skin peptides could produce proteolytic enzymes that could inhibit the antimicrobial activity by degrading skin peptides,

although proteases and other possible mechanism of resistance of pathogens to skin peptides have not been previously investigated.

1.4 Association of Impaired Immune Defences with Life Stage

Immune systems of larvae and adult frogs are different with regard to responses to invading pathogens. The responses of the adaptive immune defences of larvae are impaired compared to adults because of the low efficiency of antibodies and absence of expression of MHC I proteins (Flajnik *et al*., 1986, Flajnik *et al*., 1987, Pross and Rowlands, 2005). Such regulations of the immune defences are required to prevent autoimmune processes against the tissues that emerge in metamorphosis such as lung tissues, skin dermis and in other organs, although they increase susceptibility to intracellular pathogens such as *Ranavirus* (Carey et al., 1999).

As secretion of skin antimicrobial peptides from granular glands is fully developed after completion of metamorphosis, the innate immune defences of larvae may also be different to those in adults. The resistance of larvae to extracellular pathogens suggests that some other secretion mechanism might exist in place of granular glands. Some experimental studies have shown that small serous epidermal glands of tadpoles could secrete peptides (Liem, 1961, Delfino, 1991, Richards, 1992, Delfino *et al*., 2007). Synthesis of peptides in the skin of the Magnificent Tree Frog (*Litoria splendida*) tadpoles starts well before the onset of metamorphosis (Wabnitz *et al*. 1998). Woodhams (2003) collected skin secretions from tadpoles of three Australian species: *Litoria nannotis*, *Litoria genimaculata* and *Mixophyes shevilli* for studies with chytrid fungi but did not analyse them chemically. Secretions from tadpole skins require further characterisation in order to establish their role in innate immunity.

1.5 Frog Stressors

Stress is the effect of any force which tends to extend any homeostatic or stabilizing process beyond its normal limit, at any level of biological organization (Esch *et al*., 1975). Its cause, the stressor, may be either an anthropogenic or environmental, but such factors are not labeled "stressors" unless a stress (effect or response) is identified. "Anthropogenic stressors" of frogs may include habitat fragmentation, global climate change, pollution and captivity (Drost and Fellers, 1996, Beecher, 2006, Alford *et al*., 2007, Davidson and Knapp, 2007). "Environmental stressors" of frogs include temperature fluctuation, hydric conditions, diet, social and species interactions, ecosystem interactions and disease (Carr *et al*., 1976, Wright *et al*., 1999, Relyea and Mills, 2001, Glennemeier and Denver 2002). Responses to different stressors involve changes in physiological processes that act to adapt and regain homeostasis.

Since responses to stressors may affect susceptibility to disease and the worldwide decline of frogs, some studies have been undertaken to investigate how they could influence the immune defences. It has been found that frogs have a hormonal mechanism conserved among different vertebrates that includes elevation of corticosteroid hormones in response to a variety of environmental and anthropogenic stressors, which in turn could affect many different physiological functions of importance for survival such as immune and reproductive functions (Rollins-Smith *et al*., 1997, Wright *et al*., 1999, Hayes *et al*., 2003). Corticosteroids can influence the immune responses in various ways including reducing the number of lymphocytes (Rollins-Smith *et al*., 1997), altering immune cell trafficking, leukocyte infiltration into sites of inflammation (McEwen *et al*. 1997, Chrousos, 1998, Marques-Deak *et al*., 2005), synthesis of skin peptides (Simmaco *et al*., 1997, Miele *et al*., 1998, Simmaco *et al*., 1998) and expression of MHC I and II proteins (McEwen *et al*. 1997, Chrousos, 1998, Marques-Deak *et al*., 2005). In

general, they are thought to cause suppression of immune responses (immune-suppression) and thereby increase susceptibility to invading pathogens.

1.6 Stress Mediated Suppression of Skin Antimicrobial Peptides

In response to a variety of environmental and anthropogenic stressors, frogs secrete corticosteroids that can inhibit synthesis and release of antimicrobial peptides (Rollins-Smith *et al*., 1997, Simmaco *et al*., 1997, Miele *et al*., 1998, Simmaco *et al*., 1998). Inhibition of skin peptides mediated by corticosteroids involves increased activity of inhibitors of the NF-κB transcription factor, which is involved in the activation of genes encoding for skin peptides (Miele *et al*., 1998, Mangoni *et al*., 2001). At low environmental temperature, the total amount of recovered peptides of *L. genimaculata* is significantly reduced (Woodhams, 2003). Gibble (2006) found that the efficiency of skin peptides of *X. laevis* has been significantly reduced after exposure to atrazine. The presence of pathogens may also affect synthesis of skin peptides as the synthesis of skin peptides by frogs infected with chytrid fungi is significantly increased (Woodhams, 2003). Bathing frogs in bacterial cultures caused a significant increase in synthesis of skin peptides by *Bombina orientalis* and *Rana esculenta* frogs except when they had been treated with corticosteroids (Miele *et al*., 1998, Simmaco *et al*., 1998).

Other stressors may induce the release of skin peptides from granular glands. Davidson *et al*. (2007) showed that exposure of *Rana boylii* frogs to the carbamate pesticide carbaryl, which is known to cause prolonged stimulation of release of skin peptides from granular glands, caused a significant decrease in total amount of bioactive peptides on skin surface. Alternatively, it might be also possible that an overall response to stressors could trigger secretion of

catecholamines that in turn could increase the release of skin peptides from granular glands (Davidson *et al*., 2007).

Although stressors could inhibit skin peptides and increase susceptibility to opportunistic pathogens, their effects on frogs have not been analysed *in vivo*. Further progress in understanding the synergism between pathogens and stressors in disease development could be obtained by analyses of skin antimicrobial peptides and susceptibility to disease in studies including the simultaneous exposure of frogs to pathogen and stressor.

Inhibition of skin peptide may also occur naturally, immediately after completion of metamorphosis. The high concentrations of corticosteroids in blood of metamorphs associated with the avoidance of early activation of the immune responses that could potentially trigger autoimmune reactions against newly emerged tissues can also inhibit the synthesis skin peptides (Rollins-Smith *et al*., 1997, Simmaco *et al*., 1997, Rollins-Smith, 1998, Simmaco *et al*., 1998). Inhibition of skin peptides could be directly responsible for the high susceptibility of metamorphs to invading pathogens (Woodhams, 2003). This, however, has not been tested experimentally.

1.7 Objectives

Amongst different diseases, bacterial dermatosepticemia is the most common disease in many different frog species (Taylor *et al*., 2001). As bacteria enter the body through the skin, disease development depends on pathogen virulence and environmental factors but is modulated by the immune defences of the host (Glorioso *et al*., 1974, Pearson, 1998). The responses of adaptive

and humoral innate immunity are slow to evolve to provide protection from invading bacteria (Green and Cohen, 1977, Simmaco *et al*., 1998, Carey *et al*., 1999). This thesis was undertaken to explore whether skin peptides could have a role in protecting frogs from bacterial pathogens and how different factors such as phylogeny of frogs, pathogens, low environmental temperature, life stage and pesticides could influence their production and activity. The frog species used were the aquatic African Clawed Frog (*X*. *laevis*) and three semi-aquatic native Australian *Litoria* species that were naturalized in New Zealand through introductions in the nineteenth century: Green and Golden Bell Frogs (*L*. *aurea*), Southern Bell Frogs (*L*. *raniformis*), and Brown Tree Frogs (*L*. *ewingii*).

The specific objectives of the study were:

1) To characterise the activity of skin peptides of different frog species against different bacterial pathogens *in vitro*.

2) To determine whether low environmental temperature affects the production and activity of skin peptide of frogs.

3) To analyse how the activity of skin peptides differs with the life stage of frogs.

4) To determine whether the extracellular products of bacterial pathogens could modulate the activity of skin peptides *in vitro*.

5) To investigate relationship between activity of skin peptides and resistance to disease in an animal model that includes exposure to pathogens.

6) To determine whether the exposure to pesticide carbaryl could modulate the production and activity of skin peptides, and resistance to disease of frogs.

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Chapter 2: Activity of Skin Peptides Against Bacterial Pathogens

2.1 Introduction

Bacterial dermatosepticemia is a fatal infectious bacterial disease of frogs. Most frequently it is caused by the bacterium *Aeromonas hydrophila* (Hunsaker and Potter, 1960, Nyman, 1986, Pearson *et al*., 2000, Taylor *et al*., 2001) but some other bacterial species including *Chryseobacterium indologenes*, *Chryseobacterium meningosepticum*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus mirabilis* and *Serratia liquefaciens* have also been recorded as able to cause this disease (Glorioso *et al*., 1974, Anver and Pond, 1984, Olson *et al*., 1992, Taylor *et al*., 2001). All bacterial species associated with this disease are opportunistic pathogens that are found in the skin and gut microbiota of healthy frogs but are known to cause disease (Hird *et al*., 1981, Taylor *et al*., 1993, Taylor *et al*., 2001). Chronic stressors such as pesticides, temperature fluctuation and crowding can compromise the immune system of amphibians and increase disease susceptibility (Rigney *et al*., 1978, Carey and Bryant 1995, Taylor *et al*., 2001, Hayes *et al*., 2006). This increased susceptibility can lead to emergence of epizootic outbreaks of disease (Carey and Bryant 1995, Taylor *et al*., 2001, Hayes *et al*., 2006) associated with high mortality both in wild and captive populations (Nyman, 1986, Bradford, 1991, Taylor *et al*., 1993, Mauel *et al*., 2002).

The high mortality that is associated with this disease is attributable to bacterial toxaemia, which appears to be the proximate cause of death (Glorioso *et al*., 1974, Rigney *et al*., 1978, Taylor *et al*., 2001). Bacterial invasion through the skin leads to generalised bacteremia with

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elaboration of toxins (Glorioso *et al*., 1974, Rigney *et al*., 1978). Constant release of bacterial toxins causes extensive tissue necrosis, hyperemia, oedema and haemorrhage in many internal organs leading to death (Glorioso *et al*., 1974, Rigney *et al*., 1978). Since haemorrhage and erythema can be seen as reddening of the ventral hindlimb skin, a common public misnomer, "Red-leg syndrome", was given to the epizootic form of the disease (Emerson and Norris, 1905), though reddening of the skin can be seen in other diseases too (Taylor *et al*., 2001).

Upon bacterial entry through the skin disease development may be influenced by many different environmental and microbial factors, but is modulated by the immune defence of the host (Glorioso *et al*., 1974, Pearson, 1998). Like in other vertebrates, frog skin immunity involves adaptive and innate immune defences (Carey *et al*., 1999). The adaptive immune system includes inflammatory responses to specific antigens presented by macrophages and dendritic cells, and is mediated by specific antibodies and T-lymphocytes (Du Pasquier and Flajnik, 1990). The skin might also be protected from microbial pathogens by innate immune defences that include macrophages and neutrophils (Manning and Horton, 1982), the complement system that lyses the pathogen (Green and Cohen, 1977), natural killer cells (Horton *et al*., 1996) and antimicrobial peptides (Simmaco *et al*., 1998, Rollins-Smith *et al*., 2005). As frogs are poikilothermic animals, the inflammatory responses are too slow to develop at a sufficient rate to protect against large numbers of bacteria invading the skin (Simmaco *et al*., 1998, Carey *et al*., 1999). Similarly, humoral innate immunity, including complement mediated effects, cannot provide protection from invading bacteria in frogs (Green and Cohen, 1977, Simmaco *et al*., 1998). Thus, other mechanisms of the innate immune defences, including skin peptides, may be essential in protection from bacterial pathogens.

Chapter 2: Activity of skin peptides against bacterial pathogens

Some experimental studies have shown that secretion of antimicrobial peptides from specialised granular glands can provide protection against microbial pathogens (Simmaco *et al*., 1998, Rollins-Smith *et al*., 2002b, Woodhams *et al*., 2006). Isolated antimicrobial peptides of a variety of frog species were shown to have activity against standard reference bacterial strains (reviewed in Pukala *et al*., 2006). However, so far only peptides of the African Clawed frog (*Xenopus laevis*), including magainin 2, and peptides with amino-terminal glycine (PGL[a]), have been tested for activity against *A. hydrophila,* (Rollins-Smith *et al*., 2002a). The activity of these peptides against other pathogens, and activities of other skin peptides from all other species, have not been investigated.

Skin peptides are secreted as a mixture of different peptides that have activities that could protect against invading pathogens (Simmaco *et al*., 1998, Boman, 2000, Rollins-Smith *et al*., 2002b, Woodhams *et al*., 2006). The activity of the peptide mixture are thought to be more important in protection against bacterial pathogens than the activities of single peptide, with possible synergistic effects occurring (Westerhoff *et al*., 1995, Rollins-Smith *et al*., 2002a). As each frog species secretes its own unique set of peptides, significant variation occurs among species in the number, structure and antimicrobial activity of these peptides (Vanhoye *et al*., 2003, Conlon *et al*., 2004). Consequently the immune defences of different frog species will vary and variation in susceptibility to pathogens occur.

The objective of this study was to characterise the activities of skin peptides against different bacterial pathogens *in vitro*. Peptides were collected from four different frog species including the aquatic African Clawed Frog (*Xenopus laevis*) and three semi-aquatic native Australian *Litoria* species that were naturalized in New Zealand through introductions in the nineteenth

century: Green and Golden Bell Frogs (*L. aurea*), Southern Bell Frogs (*L. raniformis*), and Brown Tree Frogs (*L. ewingii*).

2.2 Materials and Methods

2.2.1 Collection and purification of skin peptides

Skin secretions were collected from ten adult animals of *L. aurea, L. raniformis* and *L. ewingii*, and one adult *X. laevis*, all of which were maintained in natural conditions in a vivarium at the School of Biological Sciences, University of Canterbury. Collections were made between August and October 2005. Animals were initially dosed with norepinephrine by subcutaneous injection as described by Rollins-Smith *et al*. (2002b). This method was designed to provide a standardised stimulus to induce secretions from a variety of species and to avoid the more painful electrostimulation procedure, which requires calibration for each species. Each animal was weighed, injected with 2 nmol per 1 g of body mass of norepinephrine (bitartrate salt, Sigma, St. Louis, Missouri, USA) and placed in a plastic bag containing 10 ml collection buffer per gram body weight (50 mM sodium chloride, 25 mM sodium acetate, pH 7.0) for 10 min while secretions accumulated. After removal of animals from the bags, the collection buffer containing skin peptides was acidified by addition of concentrated HCl to a final concentration of 1% to inhibit protease, filtered through a Millex HV filter unit (0.45 µm) and stored at –80ºC until lyophilisation. The lyophilised samples were redisolved to in distilled deionised water (ddH_2O) give concentration of peptides of 1 mg/ml and passed over C18-Sep-Pak cartridges (Waters Corporation, Milford, MA, USA). Peptides in the molecular weight range of 500-10,000 Da were retained on the Sep-Pak cartridges, while other peptides were discarded. Peptides bound to Sep-Pak were eluted with 70% acteonitrile, 29.9% water and 0.1% trifluoroacetic acid (TFA) and skin peptide samples were lyophilised and stored at –80ºC. At

the end of each experiment, the samples were redisolved in ddH_2O and combined to make the final stock solution. Peptide concentrations were determined by Micro BCA™ Assay (Pierce Rockford, Illinois, USA) following the manufacturer's instructions, except that bradykinin (RPPGFSPFR) (Sigma Chemical, St Louis, USA) was used to establish standard curves (Rollins-Smith *et al*., 2002b). To avoid problems with proteolysis, all peptide solutions were stored at –80ºC.

2.2.2 Protease digestion

The peptide digests were generated to provide a negative control for studies of the activity of the skin peptides. The peptide mixtures (1 mg/ml) were incubated with pronase E, a protease mixture that degrade peptides completely (Sigma Chemical, St Louis, USA), at a concentration of 0.5 mg/ml in ammonium phosphate buffer (pH 7.0) at 37ºC for 20 h. After digestion, the protease was inactivated by heating at 90 ºC for 10 min.

2.2.3 Analyses of skin peptide profiles

The liquid chromatography mass spectrometry (LC-MS) analyses of the samples of the natural peptide mixtures were carried out at Chemistry Department University of Canterbury (Christchurch, New Zealand) using Waters 2790 HPLC system. Peptide solutions analysed had a concentration of 0.1 mg/ml in ddH_20 with 0.1% formic acid. The separation was carried out using a Zorbax SB-C18 analytical column (5 µ, 2.1 x 250 mm). Mobile phase A was 0.5 % formic acid in ddH_2O and mobile phase B was 0.5 % formic acid in acteonitrile. The gradient consisted of a 40 minute run, following a solvent gradient comprised of the following steps: two minutes at 90% A and 10% B followed by a linear gradient to 25% A and 75% B over a period of 12 minutes, an isocratic hold at 25 A% and 75% B for ten minutes then a linear

gradient to 100% B over two minutes. 100% B was maintained for four minutes before the solvent ratio was returned to 90% A and 10% B in two minutes. The 90% A and 10% B was held for eight minutes to allow the column to re-equilibrate. Detection of molecular ions was undertaken using the Micromass-LCT-TOF mass spectrometer equipped with an electrospray ionisation (ESI) probe. A probe voltage of 3,200 V at 150°C, a source temperature of 80°C, nebuliser gas flow of 160 L/hr and desolvation gas flow of 520 L/hr were used. Samples were analysed with a cone voltage of 25 V unless otherwise stated. This system was controlled by MASSLYNX (Version 4.0) software. Skin peptides were identified by comparing their molecular masses with previously published masses of peptides of *X. laevis* and three *Litoria* species: *L. raniformis, L. aurea,* and *L. ewingii* (Giovannini *et al*., 1987, Moore *et al*., 1991, Steinborner *et al*., 1997, Rozek *et al*., 2000).

The liquid chromatography mass spectrometry/ mass spectrometry (LC-MS/MS) analyses were carried at The Centre for Genomics & Proteomics, The University of Auckland (Auckland, New Zealand) using the Ultimate LC system (LC Packings/Dionex). The conditions were optimised by two LC-MS analyses. Peptide solutions analysed had a concentration of 0.1 mg/ml in ddH_20 with 0.1% formic acid. The separation was carried out using a Zorbax 300SB C18 analytical column (0.3mm X 100 mm, StableBond (300Å). Mobile phase A was 0.1 % formic acid in ddH_2O and mobile phase B was 0.1 % formic acid in acteonitrile. The gradient applied was as follows: from 0 to 5 min 95% A and 5% B, from 5 to 59 min 40% A and 60% B, from 59 min to 70 min 95% B and 5% A and from 70 to 85 min 95% A and 5% B. Detection of molecular ions was undertaken using a QSTAR XL electrospray quadrupole/ time-of-flight mass spectrometer (Applied Biosystems, Foster city, CA, USA). The instrument was set to perform a TOF-MS scan from 300 to 1800 m/z for 1 second, followed by two products ion

scans from 70-1600 m/z (one 1 second, the second 2 seconds) on the two most abundant ion species (double and triple charged) using the "Enhance All" feature. The data were acquired using Analyst QS software.

The assignment of molecular masses of fragments obtained by peptides fragmentation to the primary sequence of the peptides stored in database was carried out by using Mascot search engine that include probability based algorithm (http:/www.matrixscience.com, Perkins *et al*., 1999). In Mascot, the score for an MS/MS match is based on the absolute probability (P) that the observed match between the experimental data and the database sequence is a random event. The reported peptide fragment ion's score is –10Log(P), where P is the probability that the observed match is random event and peptides scores are derived from ions scores as non-probabilistic peptide hits. The identities of peptide sequence were determined by using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1997) at the National Centre for Biotechnology Information (NCBI) web site (http:// www.ncbi.nlm.nih.gov). Identification was carried out by using the following criteria: non-redundant NCBI protein database, *X*. *laevis* and other lobe-finned fish and tetrapod clade as taxonomic category, ion score cut at 20, no enzyme used, peptide mass tolerance : ± 0.2 Da, fragment mass tolerance: ± 0.1 Da, variable modifications including amide (C-term), deamidation (NQ), oxidation (M), pyro-glu (N-term Q). Information on peptides fragments provided in appendices A–D.

2.2.4 Bacterial cultures

All bacterial isolates were obtained from frog pathogen librarium, kept at the School of Biological Sciences, University of Canterbury (Christchurch, New Zealand). The collection includes different bacterial isolates collected from the hearts of septicemic animals during an

extensive epidemiological survey between 2002 and 2006 (Barribeau, 2007). The bacterial isolates used for analyses were collected as follows. Isolates of *A. hydrophila*, *C. freundii*, *P. aeruginosa* and *S. liquefaciens* were collected from captive *X. laevis* in 2003, and the isolate of *C. meningosepticum* was collected from captive *L. aurea* in 2002. The skin isolate of saprophyte *Lactococcus lactis* was collected from captive *L. raniformis* in 2004. In addition to these isolates, isolates of *A. hydrophila*, *K. pneumoniae* and *P. mirabilis* were collected from *L. ewingii* taken from a site in Oxford Forest, New Zealand, in December 2005 at time of severe mortality outbreak. During bacterial isolation, the septicemic animals that were no longer bright, alert, and active were euthanized by immersion in a humane euthanizing agent of 3% aqueous solution of chloral hydrate, and after that were dissected under aseptic conditions. Small pieces of hearts (approx. 0.5 mg) were taken and cultured in tryptone soya broth (TSB, Oxoid, Basingstoke, UK). All cultures were incubated aerobically for 24h at 30°C. The broth cultures were then plated onto tryptone soya agar (TSA, Oxoid, Basingstoke, UK) and incubated under the same conditions. Pure colonies were examined for shape and arrangement of cells by Gram staining and identified at phenospecies level using three commercial aerobic bacterial identification systems: Microbact 24E (12E/12A + 12B, Medvet diagnostics, Thebarton, SA, Australia), RapID CB plus and RapID NF plus (Lenexa, Kansas, USA). After identification they were stored on TSA slants at 4 ºC and on cryobeads at –80ºC. The standard reference strain *Escherichia coli* (ATCC25922) was purchased from American Type Culture Collection (University Boulevard, Manassas, USA).

2.2.5 Antibacterial assays

The activities of peptide mixtures were tested using growth inhibition assays for aquatic bacteria (Rollins-Smith *et al*., 2002a). Overnight colonies of each isolate were selected from

agar, transferred to soya tryptone broth and incubated for 8 h. Following incubation, the bacterial suspension was adjusted with phosphate buffered saline to achieve turbidity of 0.5 McFarland standard (corresponding to 1.5 x 10^8 colony forming units (cfu) per ml), and the adjusted suspension was used as inoculum. For the growth inhibition assay, 50 µl of Muller-Hinton broth with a bacterial concentration of 1 x10^6 cfu was plated into each well of a 96-well microtiter plate, and 50 µl of peptide was then added to each well, in serial dilutions (500, 250, 125, 62.5, 31.2, 15.6, 7.8 and 3.9 µg/ml). The exceptions were non-peptide wells, which received 50 µl of peptide digest instead of peptides, positive control wells, which received 50 µl of ddH_2O instead of peptides, negative controls wells that received 2 µg of the peptide antibiotic polymyxin B and negative controls that received 0.4% paraformaldehyde, on separate plates. For growth inhibition assays with *L. lactis*, soya tryptone broth was used instead of Muller-Hinton broth. The samples were incubated aerobically for 24 h at 30ºC and bacterial cell growth was measured as increased optical density at 490 nm with an ELISA plate reader. Five replicate reactions were tested for each peptide concentration and three independent assays were performed for all tested peptides. To determine Minimum Inhibitory Concentration (MIC), the means of optical density values of the wells with peptide concentrations that completely inhibited growth were compared with the optical density values of the positive control reactions with no visible growth. The MIC was defined as the lowest concentration at which no significant growth was observed as in negative control reactions with 0.4% paraformaldehyde.

The same assay was used for testing activities of single peptides. The activities of magainin 2, a single peptide of *X. laevis* (GIGKFLHSAKKFGKAFVGEIMNS) (Sigma Chemical, St Louis, USA) and aurein 2.1 a single peptide of *L. aurea* and *L. raniformis*

(GLLDIVKKVVGAFGSL.CO-NH2)(Sigma-Genosys, Castle Hill, NSW, Australia) were tested at a concentration range of 200, 100, 50, 25, 12.50, 6.25 and 3.12 µg/ml. The activities of two non-amphibian peptides: polymyxin B, a single peptide of *Bacillus polymyxa* (cyclic octapeptide) (Sigma Chemical, St Louis, USA) and cecropin A, a single peptide of insect *Hyalophora cecropia* (KWKLFKKIEKVGQNIRDGIIKAGPAVAVVGQATQIAK-NH2) (Sigma Chemical, St Louis, USA) at a concentration range of 100, 50, 25, 12.5 6.25, 3.12, 1.56, 0.78, 039 and 0.19 µg/ml.

2.2.6 Determination of MIC equivalents

The efficiency of skin peptides of ten animals of each species was measured against the isolate of *K. pneumoniae*. The average amount of recovered peptides per one animal of each *Litoria* species is only sufficient to test activity only against one pathogen. To compare efficiency of skin peptides between frog species, mean MIC equivalent per 1 g body mass, and mean MIC equivalent per cm^2 surface area of each species was calculated. An MIC equivalent is the concentration of peptides recovered from each sample after elution from the C-18 Sep-Pak cartridges (concentrated to dryness and dissolved in water) adjusted to standard 1 g body mass (gbm) or per surface area divided by experimentally determined MIC (µg/ml) for each animal (Woodhams *et al*., 2006). For example, if the total amount of peptides recovered from a 10 g animal was 50 µg and MIC was 100 µg/ml, MIC equivalent for that sample of skin secretion would be 0.05. The surface area of adult animals for calculation of MIC equivalents was calculated using an equation from McClanahan and Baldwin (1969): surface area (cm^2) = 9.90 (mass in grams)$^{0.56}$. To test for differences among species in total peptides recovered and MIC equivalents per gbm (or per surface area) Kruskal-Wallis rank tests were used, and pairwise comparisons were made using Mann-Whitney U-tests with an alpha level of 0.016, following

Bonferroni's correction. All analyses were performed with SPSS v. 12 (SPSS Inc. Chicago, Illionois, USA).

2.2.7 Analysis of production and activity of skin peptides in response to low environmental temperature

In order to obtain skin secretions from animals at different environmental temperature conditions, two groups of adult animals of *L. raniformis* were held in two different environmental chambers for four weeks in February 2005. One group of animals was held at a low environmental temperature (15ºC) while the other group of animals was used as a control group and held at optimal environmental temperature (23 ºC). Animals were housed individually in Sistema 7L polypropylene box tanks with a snap lid (350 x 200 x 100 mm). The lids of the containers had drilled ventilation holes. Each box contained two dry and moistened Hygenex paper towels. Paper towels were moistened with aged water and changed weekly. In addition to these measures, a shot glass of height 55 mm and diameter 95 mm with water was put into each box to provide additional source of water. Skin peptides were collected from each animal on the first and last day of the experiment. The quantity of skin peptides recovered and their activity against the isolate of *K. pneumoniae* were measured for each animal. Differences between two groups of animals in total recovered peptides, MIC, MIC equivalents per gbm and surface area were tested by the nonparametric Mann-Whitney U-tests.

2.3 Results

2.3.1 Comparison of skin peptide profiles

Skin peptide profiles obtained by LC-MS were markedly different among species. Molecular masses of ten different peptides of *X. laevis* matched the molecular masses of previously known antimicrobial peptides belonging to four different groups including 1) two magainin

peptides (magainin 1, 2); 2) peptide with amino-terminal glycine and carboxyl-terminal glutamine (PGQ); 3) four caerulein precursor fragments (CPF 1–4); and 4) three related peptides including xenopsin precursor fragment (XPF), levitide precursor fragment (LPF) and peptide with amino-terminal glycine and carboxyl-terminal leucinamide (PGL[a]) (Fig. 2.1A; Table 2.1). Similarly, eight molecular masses of *L. aurea* peptides matched the molecular masses of previously described antimicrobial peptides of the aurein peptide family that is common to *L. aurea* and *L. raniformis*, including aurein 2.1, aurein 2.2, aurein 2.3, aurein 2.4, aurein 2.5, aurein 3.1, aurein 3.2 and aurein 5.2 (Fig. 2.1B; Table 2.2), while nine molecular masses of peptides of *L. raniformis* matched the molecular masses of aurein 1.1, aurein 1.2, aurein 2.1, aurein 2.5, aurein 2.6, aurein 3.1, aurein 3.2, aurein 3.3 and aurein 5.2 (Fig. 2.1C; Table 2.3). A molecular mass of 1427.60 Da of a peptide of *L. ewingii* corresponded to the molecular mass of uperin 7.1, the previously described antimicrobial peptide of this species (Fig. 2.1D; Table 2.4). Significant protein hits obtained by LC-MS/MS peptides fragments analyses of the natural peptides mixtures of the all four tested species confirmed the amino acid sequences of the majority of the species specific antimicrobial peptides (Tables 2.1–2.4). Thus, natural mixtures of peptides from all four tested species contained elements matching the molecular masses of known antimicrobial peptides.

Peptide digests of all four tested species did not contain the elements matching the molecular mass of known antimicrobial peptides (Fig 2.2; Appendices E–H).

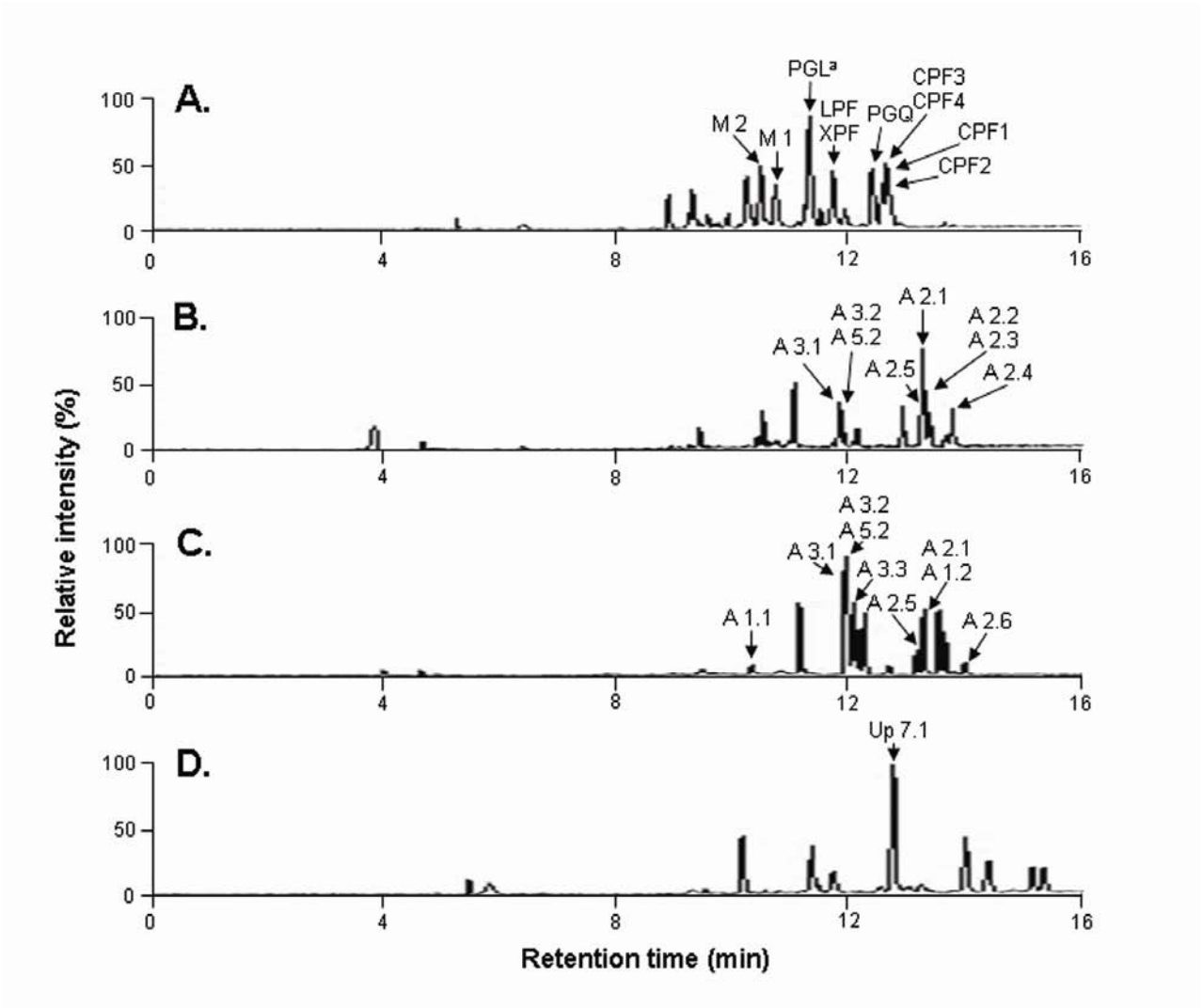

Fig. 2.1 LC-MS analyses of peptide mixtures of four different frog species. The antimicrobial peptides that were previously described are labelled as follows. (A) The antimicrobial peptides of *X. laevis* are two magainin peptides (M 1, 2), peptide with amino-terminal glycine and carboxyl-terminal glutamine (PGQ), four caerulein precursor fragments (CPF 1–4), xenopsin precursor fragment (XPF), levitide precursor fragment (LPF) and peptide with amino-terminal glycine and carboxyl-terminal leucinamide (PGL[a]). (B) Antimicrobial peptides of *L. aurea* are aureins (labelled with A followed by associated number). (C) Antimicrobial aurein peptides of *L. raniformis* are labelled as in B. (D) Up 7.1 denotes uperin 7.1, the antimicrobial peptide of *L. ewingii.*

Table 2.1 Identification of peptides of *X. laevis*.

Peptide	Relative molecular mass (Da)	Sequence
CPF 1	2614.00	GFGSFLGKALKAALKIGANALGGSPQQ
CPF 2	2690.38	GFASFLGKALKAALKIGANMLGGTPQQ
CPF 3	2602.65	GLASLLGKALKAGLKIGTHFLGGAPQQ
CPF 4	2649.05	GLASFLGKALKAGLKIGAHLLGGAPQQ
PGL[a]	1966.16	GMASKAGAIAGKIAKVALKAL-NH_2
LPF	2606.79	GWASKIGQTLGKIAKVGLQGLMQPK
XPF	2661.38	Not identified
Magainin 1	2407.50	GIGKFLHSAGKFGKAFVGEIMKS
Magainin 2	2466.58	GIGKFLHSAKKFGKAFVGEIMNS
PGQ	2455.32	GVLSNVIGYLKKLGTGALNAVLQ

Table 2.2 Identification of peptides of *L. aurea.*

Peptide	Relative molecular mass (Da)	Sequence
Aurein 2.1	1614.53	not identified
Aurein 2.2	1613.42	GLFDIVKKVVGALGSL-NH_2
Aurein 2.3	1613.42	GLFDIVKKVVGAIGSL-NH_2
Aurein 2.4	1628.46	GLFDIVKKVVGTIAGL-NH_2
Aurein 2.5	1646.49	GLFDIVKKVVGAFGSL-NH_2
Aurein 3.1	1737.59	GLFDIVKKIAGHIAGSI-NH_2
Aurein 3.2	1767.67	GLFDIVKKIAGHIASSI-NH_2
Aurein 5.2	2449.73	GLMSSIGKALGGLIVDVLKPKTPAS

Table 2.3 Identification of peptides of *L. raniformis*.

Peptide	Relative molecular mass (Da)	Sequence
Aurein 1.1	1444.48	not identified
Aurein 1.2	1479.27	GLFDIIKKIAESF-NH_2
Aurein 2.1	1614.53	GLLDIVKKVVGAFGSL-NH_2
Aurein 2.5	1646.49	not identified
Aurein 2.6	1627.65	GLFDIAKKVIGVIGSL-NH_2
Aurein 3.1	1737.59	GLFDIVKKIAGHIAGSI-NH_2
Aurein 3.2	1767.67	GLFDIVKKIAGHIASSI-NH_2
Aurein 3.3	1793.11	GLFDIVKKIAGHIVSSI-NH_2
Aurein 5.2	2449.73	GLMSSIGKALGGLIVDVLKPKTPAS

Table 2.4 Identification of peptides of *L. ewingii.*

Peptide	Relative molecular mass (Da	Sequence
Uperin 7.1	1427.60	GWFDVVKHIASAV-NH_2

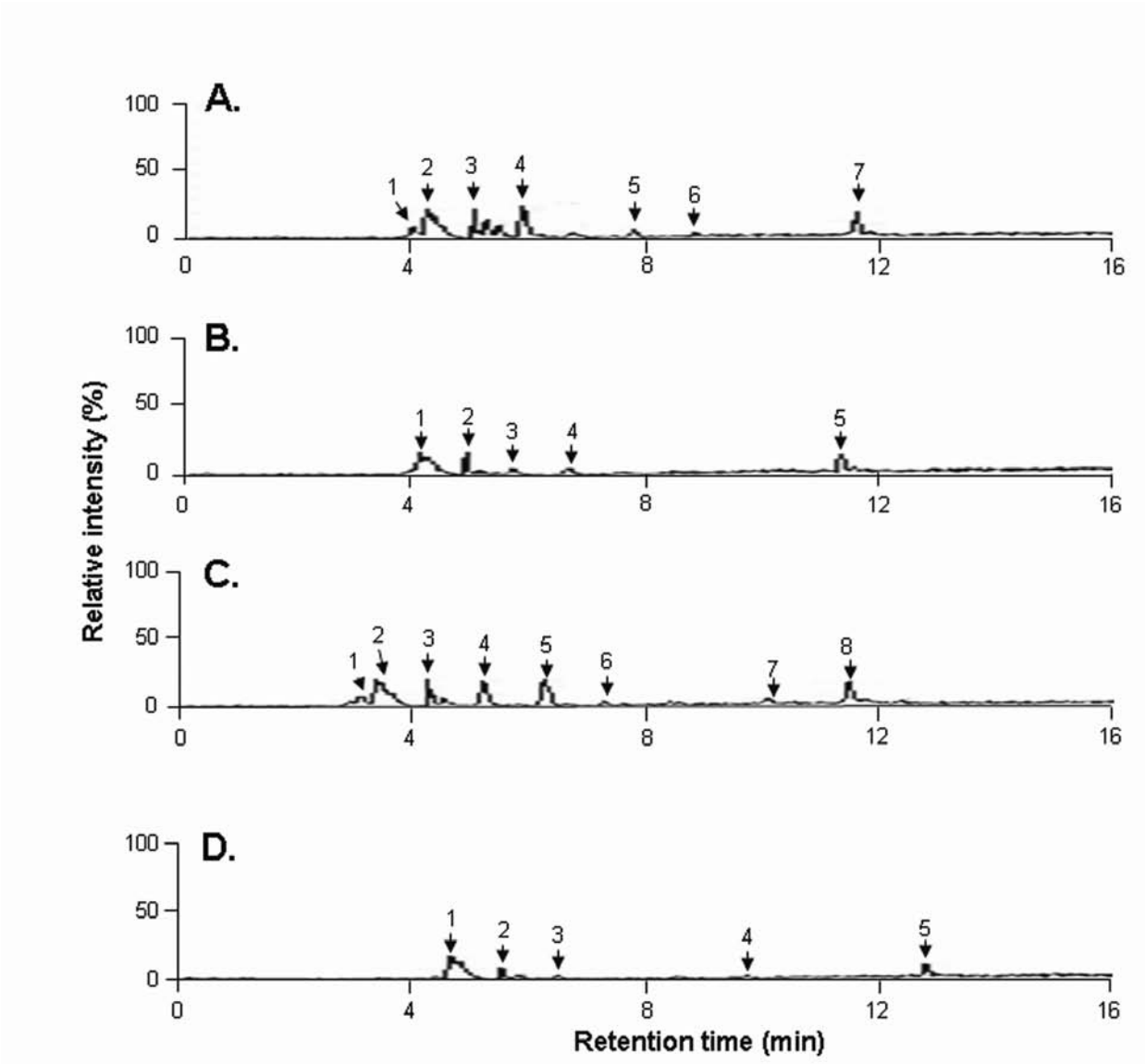

Fig. 2.2 LC-MS analyses of peptide digests of *X. laevis* (A), *L. aurea* (B), *L. raniformis* (C) and *L. ewingii* (D).

2.3.2 Antibacterial activity

The peptide mixture of *X. laevis* inhibited the growth of isolates of *C. freundii*, *C. meningosepticum*, *K. pneumoniae*, *P. aeruginosa* and *L. lactis* and *E. coli* (ATCC25922) (Fig. 2.3A; Table 2.5A). The peptide mixture of *L. aurea* inhibited the growth of isolates of *C. freundii*, *K. pneumoniae*, *P. aeruginosa* and *L. lactis* and *E. coli* (ATCC25922) (Fig. 2.3B;

Table 2.5A). The peptide mixture of *L. raniformis* inhibited the growth of the same bacteria as *L. aurea* (Fig. 2.3C; Table 2.5A). The peptide mixture of *X. laev*is was more potent against the isolate of *C. freundii*, *K. pneumoniae* and *P. aeruginosa* than the peptide mixtures of *L. aurea* and *L. raniformis* as shown by the smaller concentrations of *X. laevis* peptide mixture required to inhibit growth of these isolates (MIC values, Table 2.5A). The peptide mixture of *L. ewingii* did not inhibit the growth of any isolates completely (Figs. 2.3D and 2.6D; Table 2.5A). The only notable activity for this peptide mixture was growth inhibition of the isolates of *C. freundii* and *E. coli* (ATCC25922) at concentration of 500 µg/ml, at the rate of 75.8% and 64.9% respectively (Fig. 2.4). None of the tested peptide digests inhibited the growth of any bacteria. Two single peptides: magainin 2 and aurein 2.1 also inhibited the growth of the isolate of *C. freundii*, *K. pneumoniae*, *P. aeruginosa, L. lactis* and *E. coli* (ATCC25922) (Fig 2.5C,D; Table 2.5A). None of the tested peptide mixtures or single peptides inhibited the growth of the two isolates of *A. hydrophila* or the single isolates of *P. mirabilis* and *S. liquefaciens* (Figs. 2.6 and 2.7; Table 2.5A).

Two non-amphibian peptides polymyxin B and cecropin A inhibited the growth of different bacteria as follows. Polymyxin B inhibited the growth of two isolates of *A. hydrophila* and the isolate of *C. freundii*, *C. meningosepticum, K. pneumoniae, P. aeruginosa* and *S. liquefaciens* (Figs. 2.5A and 2.7A; Table 2.5B). Cecropin A inhibited the growth of two isolates of *A. hydrophila* and the isolate of *C. freundii*, *C. meningosepticum*, *K. pneumoniae*, *P. aeruginosa* and *P. mirabilis* (Figs. 2.5B and 2.7B; Table 2.5B). Both polymyxin B and cecropin A were not active against the isolate of *L. lactis* (Table 2.5B).

Table 2.5A Activities of skin peptides from four frog species: *X. laevis, L. aurea, L. raniformis* and *L. ewingii*. Natural peptide mixtures and single peptides were tested for growth inhibition of bacterial isolates collected from four frog species: *X. laevis* (Xl), *L. aurea* (La), *L. raniformis* (Lr) and *L. ewingii* (Le). * denotes skin saprophyte and ** denotes standard strain. NA denotes no activity.

Isolate	MIC values of peptide mixtures (µg/ml)				MIC values of single peptide (µg/ml)	
	X. laevis	*L. aurea*	*L. raniformis*	*L. ewingii*	Magainin 2	Aurein 2.1
A. hydrophila Xl	NA	NA	NA	NA	NA	NA
A. hydrophila Le	NA	NA	NA	NA	NA	NA
C. freundii Xl	31.25	62.50	62.50	NA	50.00	100.00
C. meningosepticum La	500.00	NA	NA	NA	NA	NA
E. coli (ATCC25922)**	62.50	62.50	125.00	NA	25.00	50.00
K. pneumoniae Le	125.00	250.00	250.00	NA	50.00	100.00
L. lactis Lr*	500.00	500.00	500.00	NA	100.00	100.00
P. aeruginosa Xl	62.50	125.00	125.00	NA	50.00	200.00
P. mirabilis Le	NA	NA	NA	NA	NA	NA
S. liquefaciens Xl	NA	NA	NA	NA	NA	NA

Table 2.5B Activities of non-amphibian peptides. For details see Table 2.5A.

Isolate	MIC values (µg/ml)	
	Polymyxin B	Cecropin A
A. hydrophila Le	3.12	3.12
A. hydrophila Xl	3.12	3,12
C. freundii Xl	1.56	1.56
C. meningosepticum La	25.00	12.50
E. coli (ATCC25922)**	0.78	0.78
K. pneumoniae Le	3.12	3.12
L. lactis Lr*	NA	NA
P. aeruginosa Xl	1.56	1.56
P. mirabilis Le	NA	50.00
S. liquefaciens Xl	12.5	NA

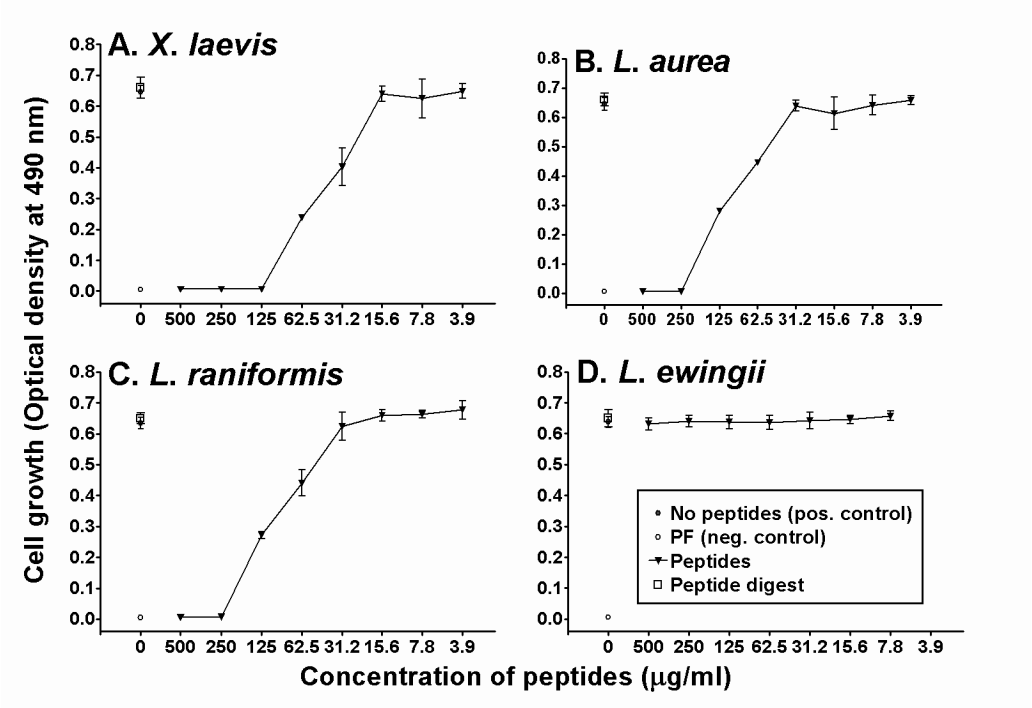

Fig. 2.3 Growth inhibition of *K. pneumoniae* by peptide mixture from *X. laevis* (A), *L. aurea* (B), *L. raniformis* (C) and *L. ewingii* (D).

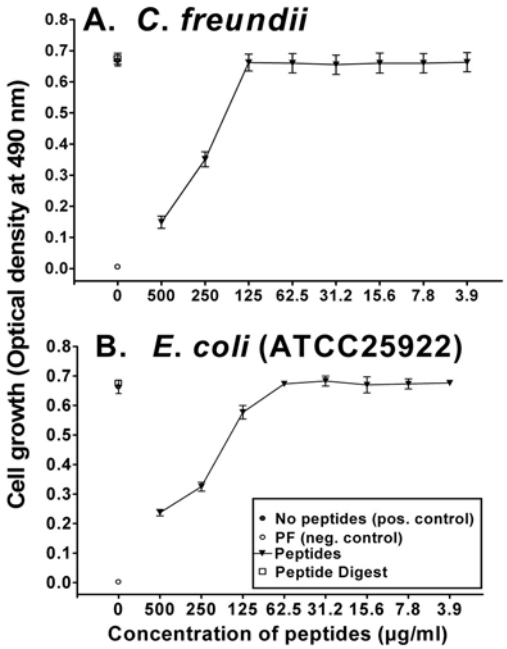

Fig. 2.4 Growth inhibition of *C. freundii* (A) and *E. coli* (ATCC25922) (B) by peptide mixture from *L. ewingii* .

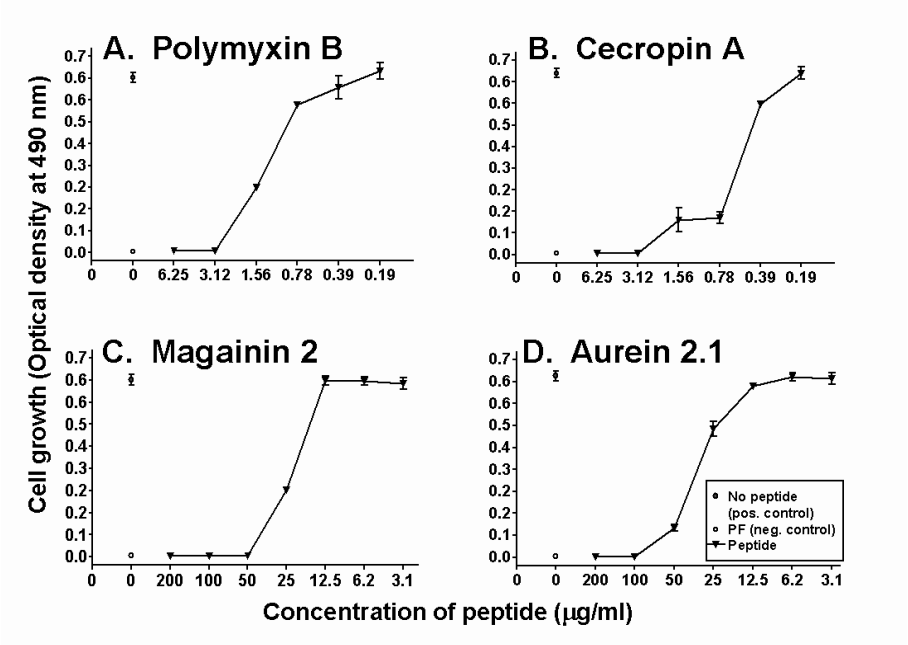

Fig. 2.5 Growth inhibition of *K. pneumoniae* by polymyxin B (A) cecropin A (B), magainin 2 (C) and aurein 2.1 (D).

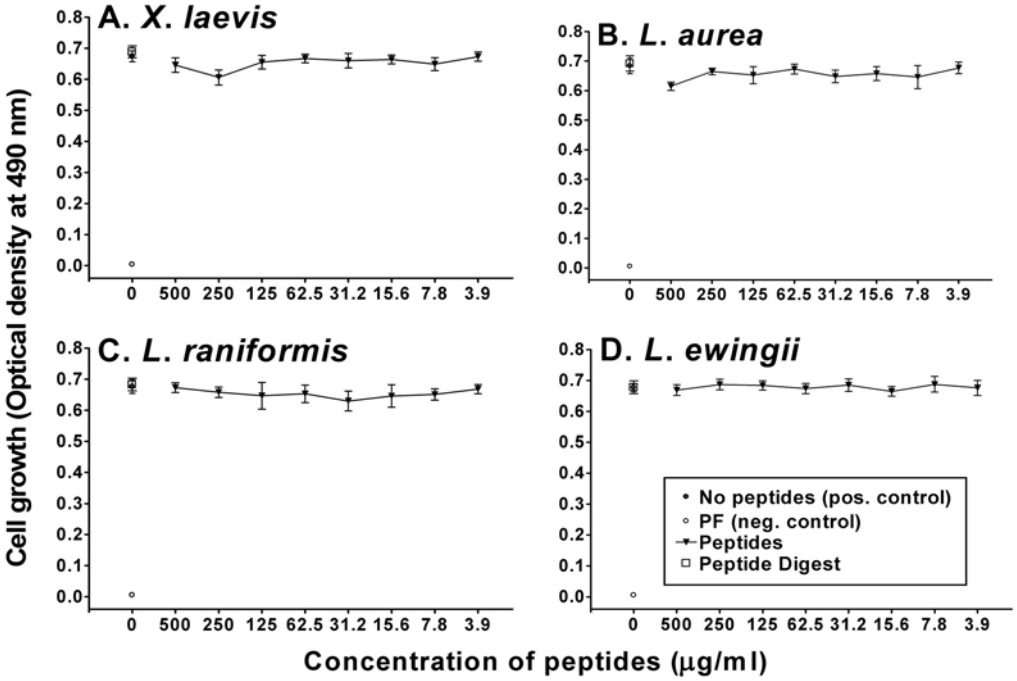

Fig. 2.6 Growth inhibition of *A hydrophila* by peptide mixture from *X. laevis* (A), *L. aurea* (B), *L. raniformis* (C) and *L. ewingii* (D).

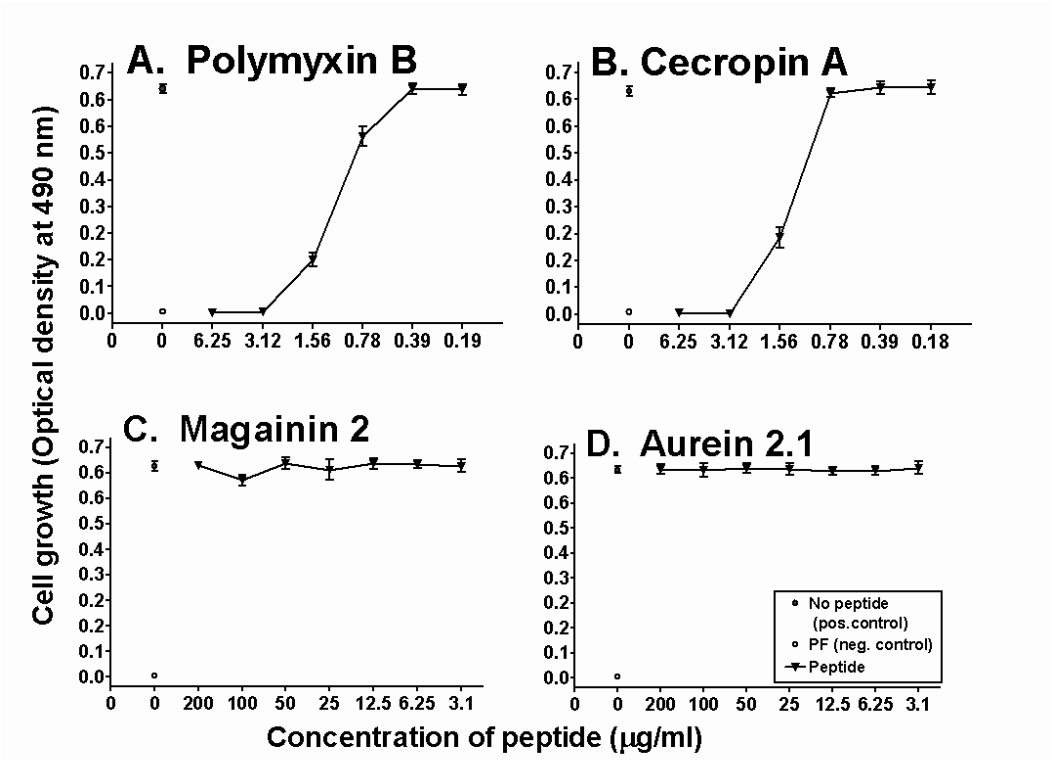

Fig. 2.7 Growth inhibition of *A. hydrophila* by polymyxin B (A) cecropin A (B), magainin 2 (C) and aurein 2.1 (D).

2.3.3 Production and efficiency of skin peptides

Among the four tested species, significant differences were found in the total amount of recovered peptide (Kruskal-Wallis rank test, $X^2_3 = 33.57$, $P < 0.05$; Table 2.6), Minimum Inhibitory Concentrations of peptides necessary to completely inhibit growth of *K. pneumoniae* (MIC (µg/ml); Kruskal-Wallis rank test, $X^2_3 = 24.08$, $P < 0.05$; Table 2.6), and in the amount of peptide active against *K. pneumoniae* on the surface area of the skin (MIC equivalents per gbm and per cm^2; Kruskal-Wallis rank test, $X^2_3 = 21.68$, $P < 0.05$ and Kruskal-Wallis rank test, $X^2_3 = 21.53$, $P < 0.05$ respectively; Fig. 2.8; Table 2.6). *X. laevis* produced the most copious peptide secretion, as the average amount of peptide per gram body mass (946.89 µg/1 gbm; Table 2.6)

was significantly higher than for the three *Litoria* species (Mann-Whitney U-test in all pair wise comparisons was significant, after Bonferroni's correction: Z = –3.78, N = 20, P < 0.016; Z statistics were identical in each test because the rank order used for their calculation did not change in the different tests; one species always occupied the highest half of the rank scores; Table 2.6). The peptide mixtures produced by *X. laevis* were more potent against *K. pneumoniae* than the peptide mixtures of *L. aurea* and *L. raniformis*, as shown by the significantly lower average minimum inhibitory concentration (MIC) of *X. laevis* (average MIC = 118.75 µg/ml; Table 2.6) compared with *L. aurea* (Mann-Whitney U-test, Z = –3.88, N = 20, P < 0.016; Table 2.6) and *L. raniformis* (Mann-Whitney U-test , Z = –4.18, N = 20, P < 0.016; Table 6). The peptide mixtures produced by *L. ewingii* were inactive (Table 2.6). A comparison of MIC equivalents demonstrated that *X. laevis* had more available active peptides per gbm and per cm^2 than *L. aurea* and *L. raniformis* (Mann-Whitney U-test in all pair wise comparisons were Z = –3.78, N = 20, P < 0.016; Fig. 2.8; Table 2.6). None of the peptide digests obtained by proteolysis of skin peptides inhibited bacterial growth.

2.3.4 Effects of temperature on production and activity of skin peptides

Before cold treatment, no difference was found between the experimental and control groups of *L. raniformis* with respect to the following variables: the average total amount of recovered peptide (Mann-Whitney U-test, Z = – 0.58, N = 14, P = 0.620; Table 2.7), the MIC of peptide mixtures against *K. pneumoniae* (Mann-Whitney U- test, Z = 0.99, N = 14, P = 1.000; Table 2.7), MIC equivalents per gbm (Mann-Whitney U-test, Z = – 0.19, N= 14, P = 0.902; Table 2.7) and MIC equivalents per cm^2 (Mann-Whitney U-test , Z = –0.48, N= 14 P = 0.710; Table 2.7). After cold treatment, the average total amount of recovered peptide and the MIC equivalents per gbm and per cm^2 for the experimental group were smaller than for the control group (Mann-

Whitney U-test, Z = –2.75, N = 14, P < 0.05 for total recovered peptides, Z = –2.49, N = 14, P < 0.05 in pairwise comparisons of both MIC equivalents; Fig. 2.9; Table 2.7) while the average MIC of peptide mixtures against *K. pneumoniae* was not different (Mann-Whitney U-test, Z = 0.63, N = 14, P = 710; Table 2.7). None of the peptide digests obtained by proteolysis of skin peptides inhibited bacterial growth.

Table 2.6 Production of skin peptides by four different species and efficiency against *K. pneumoniae*.

Species	N animals	Total peptides per 1gbm +/-SD	Average MIC (µg/ml))+/- SD	MIC equivalents per 1gbm +/-SD	MIC equivalents per surface area (cm^2)+/-SD
X. laevis	10	946.89 +/- 113.96	118.75 +/- 19.76	8.533 +/- 3.780	6.625 +/- 3.597
L. aurea	10	76.32 +/- 6.08	237.50 +/- 39.53	0.336 +/- 0.103	0.160 +/- 0.045
L. raniformis	10	67.90 +/- 7.05	275.00 +/- 79.06	0.272 +/- 0.028	0.141 +/- 0.006
L. ewingii	10	117.40 +/- 21.20	No activity	0.000+/- 0.000	0.000 +/- 0.000

Table 2.7 Production of skin peptides of *L. raniformis* maintained at 15ºC and efficiency against *K. pneumoniae*.

Group	N animals	Total peptides per 1gbm +/-SD before treatment	Total peptides per 1gbm +/-SD after treatment	MIC (µg/ml))+/- SD before treatment	MIC (µg/ml))+/- SD after treatment	MIC equivalents per 1gbm +/- SD before treatment	MIC equivalents per 1gbm +/-SD after treatment	MIC equivalents per cm^2 surface area +/-SD before treatment	MIC equivalents per cm^2 surface area +/-SD after treatment
Treatment	7	75.45 +/- 9.51	57.15 +/- 6.59	232.14 +/- 47.25	232.14 +/- 47.25	0.347 +/- 0.133	0.260+/- 0.083	0.176 +/- 0.069	0.131 +/- 0.043
Control	7	73.59 +/- 7.70	68.29 +/- 5.15	232.14 +/- 47.25	214 +/- 60.99	0.330 +/- 0.099	0.346+/- 0.113	0.180 +/- 0.062	0.188 +/- 0.076

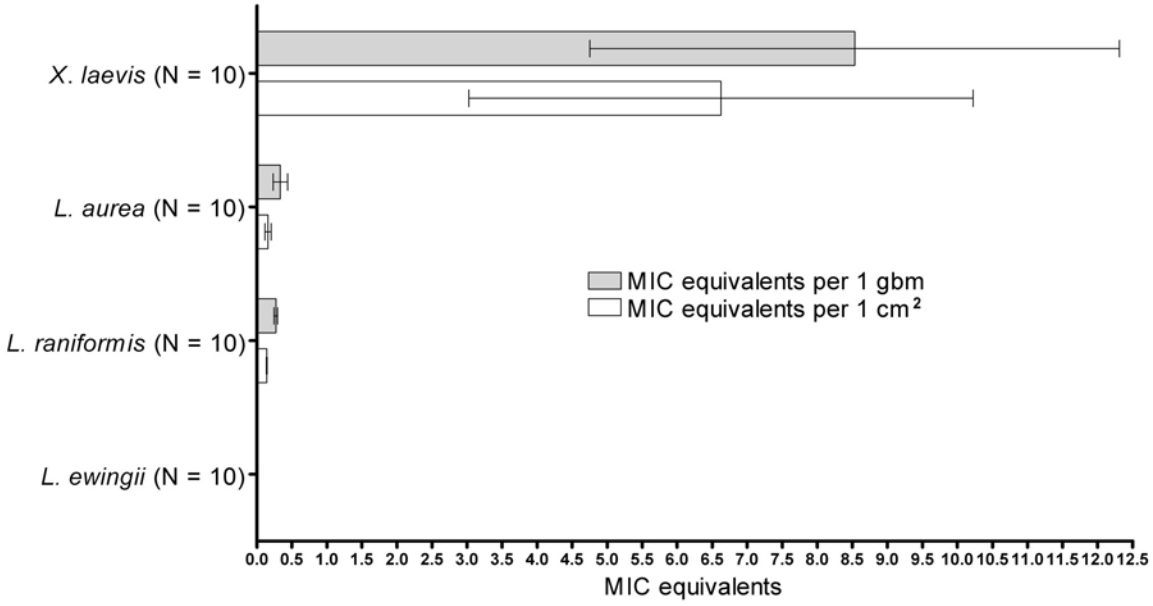

Fig. 2.8 Comparison of MIC equivalents of four different species. If value bar is not shown, the value is equal to 0.

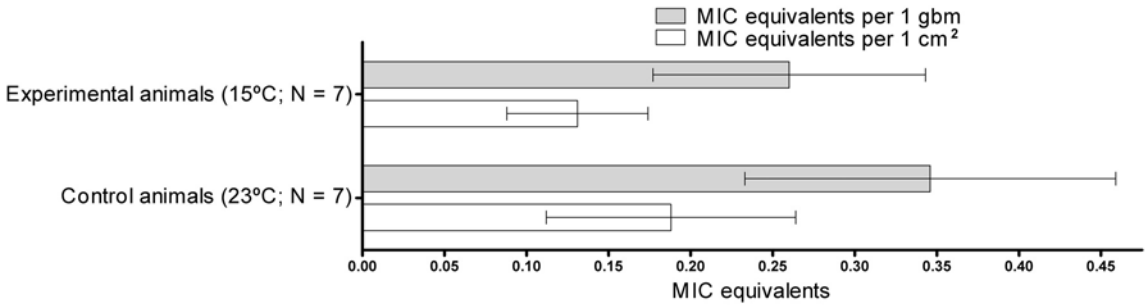

Fig. 2.9 Comparison of MIC equivalents of two groups of *L. raniformis* held at two different environmental temperatures.

2.4 Discussion

The results show that peptide defences might provide direct protection from some opportunistic bacterial pathogens associated with bacterial dermatosepticemia. Most importantly, it was observed that natural peptide mixtures and single peptides of three species including *X. laevis* and two *Litoria* species, *L. aurea* and *L. raniformis*, inhibited the growth of three different pathogens including *C. freundii*, *K. pneumoniae* and *P. aerugionsa*, and the peptide mixture of *X. laevis* also inhibited the growth of *C. meningosepticum* (Figs. 2.3A,C and 2.5C,D; Table 2.5A). Thus, the skin peptides of three species had activities against certain bacterial pathogens *in vitro*, and they might be expected to provide significant protection *in vivo*.

2.4.1 Interspecies variations in activities of skin peptides

The significance of skin peptide activities in protection of amphibians from different bacterial pathogens may depend on a number of factors. It appears that the aquatic species *X. laevis* might have more efficient immune defences in the terms of production and activity of skin peptides than the three terrestrial *Litoria* species. *X. laevis* produced a significantly greater amount of active skin peptide on the skin surface than *L. aurea* or *L. raniformis*, while *L. ewingii* did not have active peptides (Fig. 2.8; Table 2.6). Similarly, the peptide mixture of *X. laevis* was able to inhibit growth of several bacteria to a greater extent than the peptide mixtures of the three tested *Litoria* species (Fig. 2.3A,D; Table 2.5A). The greater efficiency of the *X. laevis* peptide mixture could have been due, at least in part, to its greater diversity compared with those of the three *Litoria* species, as observed by LC-MS analyses (Fig. 2.1). The potential importance of peptide diversity is supported by the fact that *L. ewingii*, whose

skin peptide mixture did not have activity against bacterial pathogens (Figs. 2.3D and 2.6D; Table 2.5A), has the smallest diversity of skin peptides (Fig. 2.1; Table 2.4) and is more susceptible to disease than the other *Litoria* species *L. raniformis* in my study region (Jenkins, 2004, Schadich, unpubl. data). Peptide diversification could therefore also be an adaptation mechanism to opportunistic bacteria in some habitats.

2.4.2 Resistance of *A. hydrophila*, *P. mirabilis* and *S. liquefaciens* to skin peptides

Three different pathogens, *A. hydrophila*, *P. mirabilis* and *S. liquefaciens*, were resistant to natural mixtures of skin peptides from all four frog species, showing that skin peptides cannot provide protection against all pathogens (Figs. 2.6 and 2.7; Table 2.5A). Some animal and human isolates of these pathogens have ability to develop resistance to different kinds of antimicrobial peptides by secretion of proteases or by modification of the polysaccharide components of their outer membrane (Storm *et al*., 1977, Smeianov *et al*., 2000, McCoy *et al*., 2001, Rosenfeld *et al*., 2006). *Aeromonas*, *Proteus* and *Serratia* spp. are normal components of skin microbiota of healthy frogs (Hird *et al*., 1981), so resistance to skin peptides may have co-evolved with their host (Boman 2000, Woodhams *et al*., 2007). The symbiotic bacterial species that are present on skin might be regulated by microbial antagonism, as some bacterial species have been shown to inhibit the growth of other bacterial species by chemical or competitive means (Pearson 1998, Woodhams *et al*., 2007). This regulation may be disrupted by stress associated with seasonal changes in environmental conditions, pollution and crowding, all of which could favour bacterial growth, including growth resulting from over-stimulation of secretion of skin peptides and consequent removal of antagonistic bacteria (Hazen, 1979, Harris *et al*., 2006, Ashcroft *et al*., 2007, Ryu *et al*., 2008). Systematic dissemination of bacteria that may pass through the skin may still be prevented by the innate immune system,

including macrophages in the skin, lymphatic sinuses and spleen which have a high capacity to eliminate pathogens (Pearson, 1998, Taylor *et al*., 2001, Hayes *et al*., 2006). Thus, disease development caused by *A. hydrophila*, *P. mirabilis* and *S. liquefaciens* may be associated with host-pathogen relations that are influenced by regulatory mechanisms other than skin peptides. These mechanisms could include microbial antagonism in skin microbiota and macrophages of the innate immune system.

2.4.3 Effects of temperature on production and activity of skin peptides

Production of skin peptides by *L. raniformis* was significantly reduced at low temperature (Fig 2.9; Table 2.7). Some environmental factors have been shown to inhibit synthesis and release of skin peptides (Rollins-Smith *et al*., 2002b). Specifically, in response to changes in environmental conditions and anthropogenic stressors, frogs and other amphibians produce corticosteroid hormones which can have an immunosuppressant effect including inhibition of skin peptide production (Simmaco *et al*., 1998, Carey *et al*., 1999). Synthesis and release of skin peptides by the hibernating wood frog, *Rana sylvatica*, were completely inhibited by lowering environmental temperature to 5 ºC (Matutte *et al*., 2000). Thus, seasonal susceptibility to disease might be a consequence of environmental conditions affecting skin defences and pathogen virulence (Hazen, 1979, Taylor *et al*. 2001). Future studies should focus on the possible influence on the immune response of other environmental factors including pH, ultra-violet radiation and pollutants.

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Chapter 3: Comparison of Skin Peptides of Tadpoles, Metamorphs and Adults of *L. ewingii*

3.1 Introduction

In frogs, granular glands of skin dermis are important in innate immune defences. They are the site of synthesis and storage of antimicrobial peptides (Bevins and Zasloff, 1990, Erspamer, 1994). Following injury or microbial challenge, these peptides are secreted onto skin surface to provide protection from invading microbial pathogens (Simmaco *et al*., 1998, Rollins-Smith *et al*., 2005, Woodhams *et al*., 2007).

The granular glands are fully developed only after metamorphosis, a transition period from tadpole (larvae) to adult animal (Delfino, 1991, Viertel and Richter, 1999) which might suggest the innate defences of tadpoles not be fully developed. Moreover it is known that the adaptive immune defences of tadpoles that include responses specific to pathogens are impaired (Flajnik *et al*., 1986, Flajnik *et al*., 1987, Pross and Rowlands, 2005). These facts raise the question of how the skin of tadpoles is protected from invading microbial pathogens prior to metamorphosis.

Immediately after metamorphosis the adaptive immune defences of newly metamorphed animals are suppressed by high blood levels of corticosteroids associated with metamorphic events (Rollins-Smith, 1998). Secretion of skin peptides from granular glands can also be affected by corticosteroids and therefore the skin defences of metamorphs could be suppressed (Simmaco *et al*., 1998).

Some experimental studies have shown that small serous epidermal glands of tadpoles could secrete peptides (Liem, 1961, Richards, 1992, Delfino, 1991, Delfino *et al*., 2007). Wabtntiz *et al*. (1998) demonstrated the synthesis of peptides of tadpoles of the Magnificent Tree Frog (*Litoria splendida*) in skin starts well before the onset of metamorphosis. Woodhams (2003) collected skin secretions from tadpoles for studies with chytrid fungi but did not analyse them chemically.

Secretion of skin peptides may depend on the life stage of the frogs. The aim of this study was to determine how antibacterial activity of skin peptides differs with the three different life stages: tadpoles, metamorphs and adults. Peptides were collected from tadpoles and metamorphs of *L. ewingii* and tested for activity.

3.2 Material and Methods

3.2.1 Rearing tadpoles and metamorphs

Animals used in this study were collected as recently hatched tadpoles at a pond in Oxford Forest, Canterbury in August 2007 and raised until completion of metamorphosis in a vivarium. Their developmental stages were determined using the Anstis 46-stage developmental chronology (Anstis, 2002). A photograph shows animals at two different developmental stages (Fig. 3.1). The tadpoles were kept in propylene tanks (340 x 290 x 180 mm) and progressively thinned to a final density of 1 tadpole/l of pond water to attenuate the negative impact of growth inhibitors emitted by fast-growing individuals. Their daily diet consisted of frozen endive fragments *ad libitum* once daily and Wardley's “Small fry” liquid food once per week as a protein supplement. At metamorphosis, the water levels of tanks were

reduced to 1 cm and changed weekly. After metamorphosis, metamorphs were fed adult vinegar flies, *Drosophila melanogaster*, three times per week. The water source both for tadpoles and metamorphs was municipal water that was aged, non-chlorinated and filtered. Two adult animals were obtained from the captive colony of *L. ewingii* kept in the vivarium.

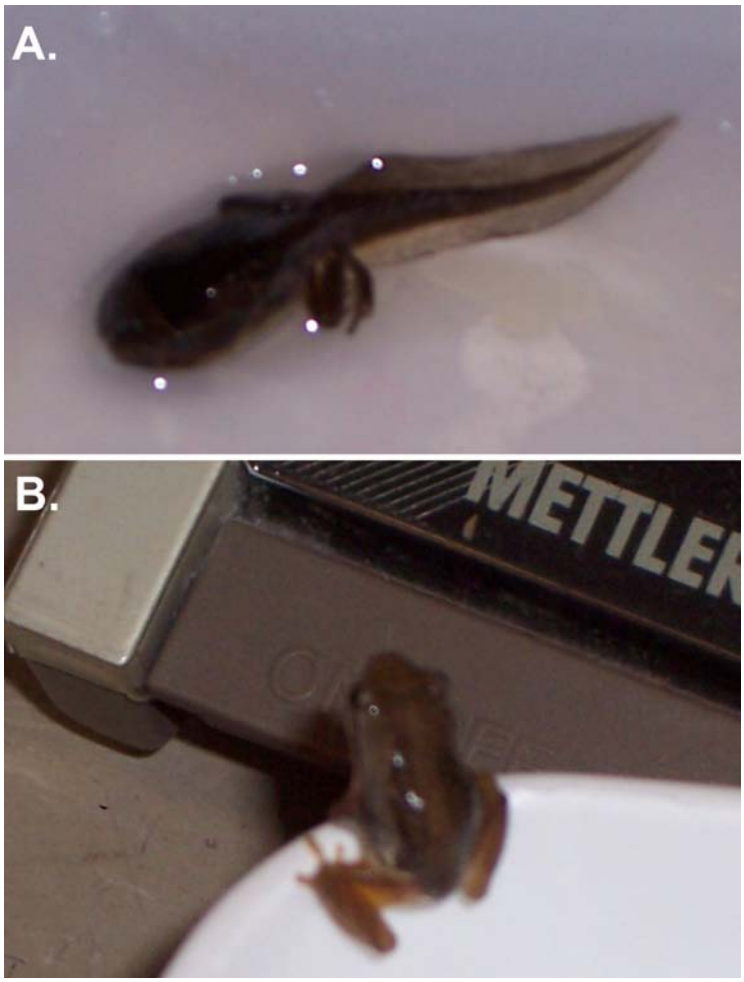

Fig. 3.1 Tadpole at stage 41 (A) and metamorph at stage 46 (B).

3.2.2 Induction, collection and purification of skin peptides

Skin peptides were induced and collected from ten tadpoles (stage 41), ten metamorphs (stage 46) and two adults by the same procedure as described in section 2.2.1 with two exceptions. The first exception was that the dosage of norepinephrine was reduced to 1 nmol per 1 g of body mass in order to avoid negative effects of norepinephrine on tadpoles and metamorphs. The second exception was the tadpoles were dosed with norepinephrine by intramuscular injections into the dorsal tail muscle due to absence of a developed dermis while adults and metamorphs were dosed with norepinephrine as described in section 2.2.1. Purification and quantification of skin peptides was carried out as described in section 2.2.1. To obtain sufficient material for analyses, the peptide samples were pooled.

3.2.3 Protease digestion

Peptide digest of adults and metamorphs were obtained by digestion of peptides with Pronase E. Peptide digests of tadpoles were not obtained due to limited amount of recovered peptides. The procedure for peptide digestion was as described in section 2.2.2.

3.2.4 Analyses of antibacterial activity of skin peptides of tadpoles and metamorphs

The activity of peptide mixtures was measured against easily manipulated *Escherichia coli* (ATCC25922) as an indicator organism by growth inhibition assay as described in section 2.2.5 except that only peptide concentration of 500 µg/ml was tested because of limited peptide amounts. Five replicate reactions were tested for each peptide sample and two independent assays were performed. Growth inhibition was calculated using the following formula ((positive control optical density – sample optical density)/(positive control optical density)) X 100 and expressed as a percentage.

3.2.5 LC-MS analyses of skin peptides

LC-MS analysis of peptide mixtures was performed as described in section 2.2.3.

3.3 Results

3.3.1 Negative effects of peptide collection

Three tadpoles died within one hour after collection of skin peptides. The other tadpoles and all of the metamorphs and adults remained healthy without any sign of change in behaviour.

3.3.2 Amount of collected peptides

The amounts of recovered peptides of tadpoles, metamorphs and adults were 431, 761 and 688 µg respectively.

3.3.3 Skin peptide profiles of tadpoles, metamorphs and adults

Skin peptide profiles obtained by LC-MS were markedly different between tadpoles, metamorphs and adults. A molecular mass of 1427.60 Da of a peptide from adults and metamorphs corresponds to that of uperin 7.1, the species-specific antimicrobial peptide (Fig. 3.2B,C; Table 3). The molecular masses of the peptides from tadpoles do not respond to the molecular mass of any known frog antimicrobial peptide (Fig. 3.2A; Table 3). Peptide digests of adults and metamorphs did not contain elements matching the molecular mass of any known antimicrobial peptides (Fig 3.3; Appendix I).

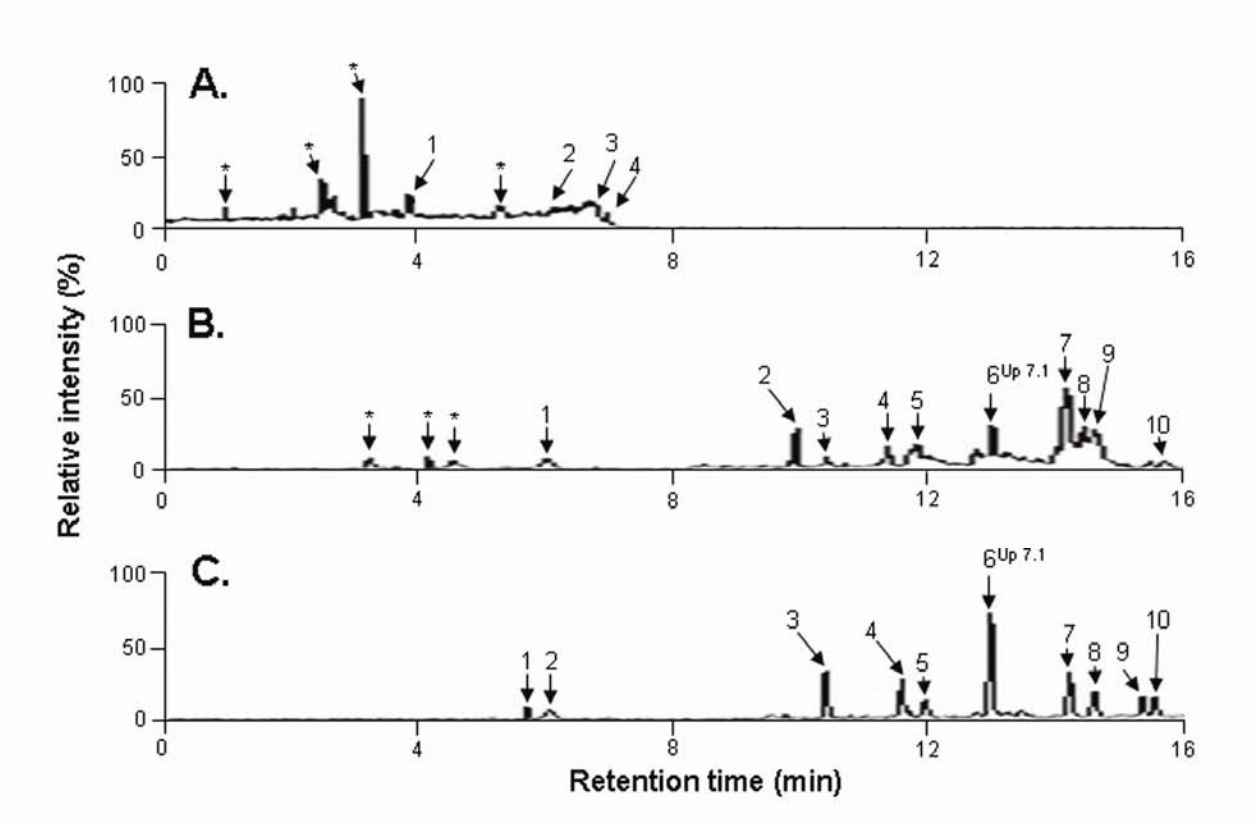

Fig. 3.2 LC-MS analyses of peptide mixtures of tadpoles (A), metamorphs (B) and adults (C) of *L. ewingii*. The numbers denotes peaks with peptides and the superscript Up 7.1 associated with the number denotes the peak with species-specific antimicrobial peptide uperin 7.1. * denotes peaks with non-peptide compounds.

Table 3 Molecular masses of peptides of tadpoles, metamorphs and adults of *L. ewingii*.

Life stage					
Tadpoles		Metamorphs		Adults	
LC-MS Peak	Relative molecular mass (Da)	LC-MS Peak	Relative molecular mass (Da)	LC-MS Peak	Relative molecular mass (Da)
1	504.84	1	1863.93	1	703.20
2	781.30	2	703.20	2	979.38
3	506.88	3	1242.62		1176.60
4	1814.22	4	1176.60	3	1177.76
		5	1468.92	4	1225.59
		6	1427.60	5	1185.63
		7	961.66	6	1427.60
			1695.26	7	817.88
		8	1924.32	8	1456.10
		9	1721.43	9	1456.10
		10	1736.37	10	1454.12

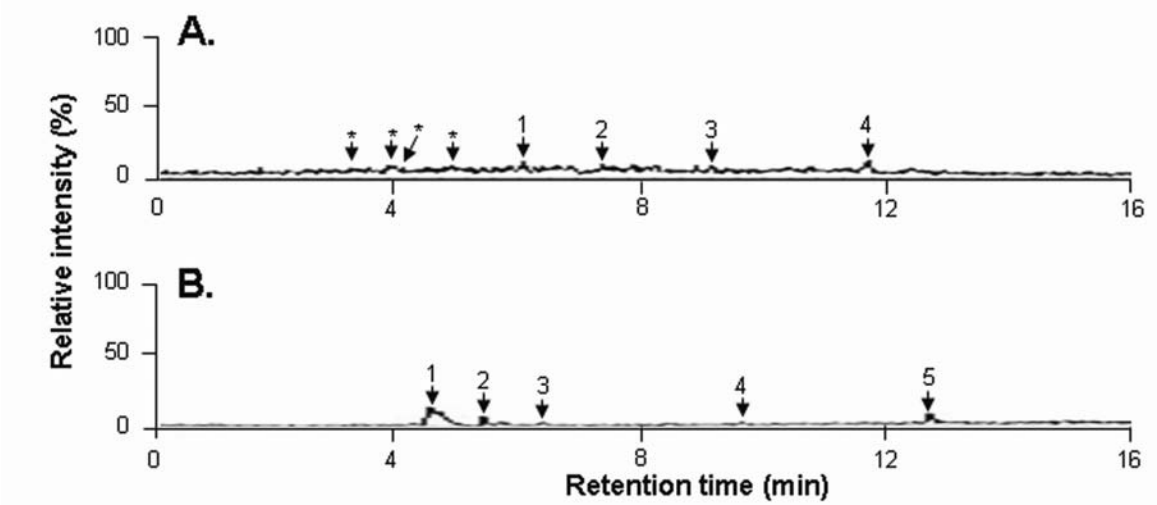

Fig. 3.3 LC-MS analyses of peptide digests of adults (A) and metamorphs (B) of *L. ewingii*. The numbers denote peaks with peptides and * denotes peaks with non-peptide compounds

3.3.4 Activity of skin peptides of tadpoles, metamorphs and adults

The peptide mixture of tadpoles did not inhibit the growth of *E. coli* (ATCC25922) (Fig. 3.4). The peptide mixtures of adults and metamorphs inhibited the growth of *E. coli* (ATCC25922) by 82.7 and 71.5% respectively (Fig. 3.4). The peptide digests of adults and metamorphs did not inhibit the growth of *E. coli* (ATCC25922) (Fig. 3.4).

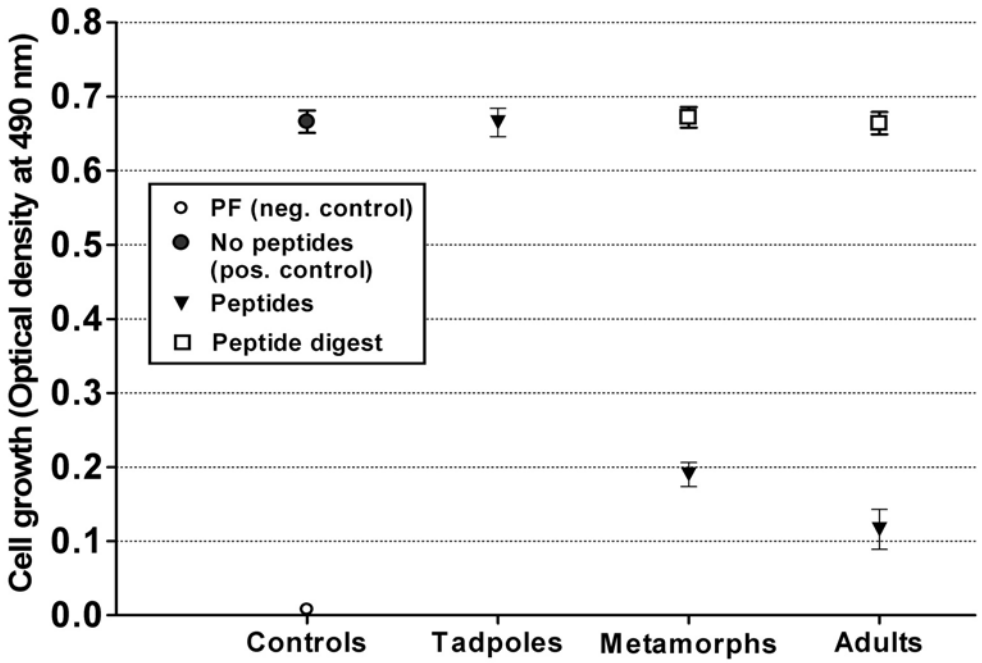

Fig. 3.4. Growth inhibition of *E. coli* (ATCC25922) by peptide mixtures of tadpoles, metamorphs and adults of *L. ewingii*.

3.4 Discussion

Analyses of antibacterial activity of skin secretions of frogs at three different stages of their life history: tadpoles, metamorphs and adults showed that this aspect of their innate immune defences is present in postmetamorphic animals, metamorphs and adults but not in tadpoles. The peptide mixture of metamorphs and adults had notable activity against *E. coli* (ATCC25922) while the peptide mixture of tadpoles was inactive (Fig. 3.4). These differences correlated with differences in peptide profiles, since the peptide mixtures of metamorphs and adults had the species-specific antimicrobial peptide present (Fig. 3.2B,C; Table 3) while that of tadpoles did not possess any antimicrobial peptide (Fig. 3.2A; Table 3) which is consistent with the absence of granular glands in tadpoles (Delfino, 1991).

Skin peptides were collected from tadpoles that have small serous epidermal glands (Fig. 3.2A; Table 3) providing evidence of a secretory mechanism exclusive of granular glands. The physiological role of these peptides is not known, but could include chemical cues to conspecifics (Fox, 1988). Since skin secretions are conserved physiological mechanisms (Erspamer, 1994), these observations on skin peptides of tadpoles of *L. ewingii* suggest avenues for future research on the role of skin peptides in tadpoles.

The skin innate defences of tadpoles do not appear to include antimicrobial peptides as indicated by absence of activity against *E. coli* (ATCC25922) (Figs. 3.2A and 3.4) although it is possible there might be extreme specificity with pathogenic bacterial strains. An innate defence that is efficient includes isolation and sequestration of pathogens by a well developed system of macrophages (Ishii *et al*., 2007), and this mechanism might be essential in protection

against pathogens. In addition, resistance of tadpoles to some pathogens could depend on presence or absence of specific skin structure such as the absence of keratinised stratum corneum in epidermis which confers resistance to the frog chytrid fungus (*Batrachochytrium dendrobatidis*) associated with disease (Berger *et al*., 1999).

The observation that skin peptides from metamorphs had antibacterial activity (Figs. 3.2B,C and 3.4) suggests a role of skin peptides in adaptation to microbes of changed habitat. Metamorphs encounter many new potential pathogens as they switch from an aquatic to a dual habitat that includes both aquatic and terrestrial habitation (Alford, 1999). The only efficient immune defence that includes macrophages is suppressed due to the high blood level of corticosteroids associated with metamorphic events (Rollins-Smith, 1998). Furthermore due to keratinisation of stratum corneum of epidermis, susceptibility to disease increases (Berger *et al*., 1999). Thus, secretion of skin peptides could be essential in protection of metamorphs against pathogens. Further studies should focus on activity of skin peptides of metamorphs against frog-specific pathogens.

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Chapter 4. Protease Mediated Inactivation of Skin Peptides

4.1 Introduction

The bacterium *Aeromonas hydrophila* and some other Gram-negative bacterial species including *Chryseobacterium indologenes, Chryseobacterium meningosepticum, Citrobacter freundii, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa* and *Serratia liquefaciens* have been associated with bacterial dermatosepticemia, a fatal infectious disease of frogs (Glorioso *et al*., 1974, Nyman, 1986, Taylor *et al*., 2001, Mauel *et al*., 2002). They are opportunistic pathogens that are normally found in the skin and gut microbiota of healthy frogs but are known to cause disease in animals with compromised immune defences (Hird *et al*., 1981, Carey and Bryant, 1995, Taylor *et al*., 2001, Hayes *et al*., 2006).

Virulence of these bacteria depends on the elaboration of various biologically active extracellular products (ECPs) (Rigney *et al*., 1978, Pearson, 1998, Pearson *et al*., 2000). Rigney *et al*. (1978) have demonstrated that bacterial ECPs with haemolytic activity are associated with extreme oedema, haemorrhage and tissue necrosis in many organs while Pearson (1998) showed that proteases are associated with tissue invasion by bacteria and suggested that because of their ability to degrade different protein components these bacteria might inhibit certain immune mechanisms.

The only effective immune defence of frog skin against invading pathogens is the antimicrobial peptides of the innate immune system. Inhibition of these peptides by the ECPs could enable pathogens to evade elimination (Simmaco *et al*., 1998, Rollins-Smith *et al*., 2002, Rollins-

Smith *et., al* 2005, Woodhams *et al*., 2007). Evidence for a role of proteolytic ECPs in the resistance of bacteria to skin peptides has been found in this study. It was shown that the bacterium most frequently isolated from diseased frogs, *A. hydrophila,* is resistant to skin peptides of four different frog species including African Clawed Frogs (*Xenopus laevis*) and three Australian *Litoria* species: *L. aurea*, *L. raniformis* and *L. ewingii*. Three other pathogens, *C. freundii*, *K. pneumoniae and P. aeruginosa* were susceptible to the skin peptides of three of these species *X. laevis, L. aurea and L. raniformis* (Rollins-Smith *et al*., 2002; described in Chapter two). To date the resistance mechanisms of frog bacterial pathogens to skin peptides have not been studied.

The aim of this study was to determine whether the bacterial ECPs with protease activity can modulate the action of skin peptides. ECPs from *A. hydrophila* and *K. pneumoniae* were tested against skin peptides of three frog different species: *X. laevis, L. aurea* and *L. raniformis*.

4.2 Materials and Methods

4.2.1 Bacterial cultures

Isolates of *A. hydrophila* and *K. pneumoniae* collected from wild *L. ewingii* were accessed from the U. C School of Biological Sciences collection of frog bacterial isolates as described in section 2.2.4.

4.2.2 Peptide solutions

Peptide mixtures of *X. laevis*, *L. aurea* and *L. raniformis*, two single peptides magainin 2 from *X. laevis* and aurein 2.1 from *L. aurea* and *L. raniformis* respectively and polymyxin B were prepared from stock solutions as described in section 2.2.1.

4.2.3 Growing bacterial cultures, production and preparation of extracellular products (ECPs)

Bacterial isolates were grown in 18 h shake culture in 50 ml of tryptone soya broth (TSB, Oxoid, Basingstoke, UK) in 100 ml Erlenmeyer flasks at 30ºC. 100 µl of this bacterial broth were inoculated into 250 ml of tryptone soya broth in 500 ml Erlenmeyer flasks and incubated as above for 36 h. Samples were removed at 0 h, 8 h, 16 h, 24 h and 36 h and examined for bacterial growth by determining the optical density at 610 nm. The bacterial suspension was then centrifuged at 10, 000 g for 20 min at 4ºC, and the supernatant filter sterilized using a 0.22 µm Millipore membrane filter. The cell free supernatant was used as a source of the ECPs. Three independent experiments were performed for each bacterial isolate.

4.2.4 Protease detection on agar media

Proteases produced by bacterial cells growing on Mueller-Hinton agar containing 10% (w/v) skimmed milk were assessed as described by Castro-Escarpulli *et al*. (2003). The bacterial broth that was used as inoculum was spread to the point of attaining single colonies and incubated at 30 ºC for 24h. The presence of a clear zone around the colonies indicated protease activity.

4.2.5 Protease activity of ECPs

Proteolytic activity of ECPs from each culture was determined as described by Khalil and Mansour (1997). Casein 1% (w/v) dissolved at in 0.1 M glycine-sodium hydroxide buffer (pH 9.6) was used as the substrate solution. One milliliter of ECP was added to 1ml substrate solution and incubated at 30ºC for 10 min. The reaction was stopped by adding 0.1ml of 1M trichloroacetic acid and left to stand for several minutes. The resultant precipitate was removed by centrifuging at 6, 000 g for 10 min at 4ºC. The absorbance of supernatant fluid was measured at 280 nm. In control reactions trichloroacetic acid was added at zero time. One unit of proteolytic activity was expressed as an increase of 0.01 in the absorbance value at 280 nm.

4.2.6 Protease Inhibitor Treatment

The ECPs of each culture were incubated at 30ºC for 1 h with the three different protease inhibitors, phenyl methyl sulfonyl fluoride (PMSF; 1 mM), ethylene diamine tetra-acetic acid (EDTA; 2 mM) and leupeptin (0.1 mM), which are specific for proteases of *Aeromonas* species.

4.2.7 Effects of temperature on protease activity of ECPs

The stability of proteases at elevated temperature was measured by heating 300 µl of the ECP of each culture to 90 ºC for 15 minutes. After heat treatment, the protease activity was measured as described above.

4.2.8 Inhibition of skin peptide activity by ECPs

The ability of the ECPs to inhibit the activity of peptide mixtures and single peptides was determined by bio-assay as described by Devine *et al*. (1999). 150 µl solutions of peptides at a

concentration of 300 µg/ml in ddH2O were incubated with 150 µl of ECPs or ECPs that had been heat inactivated at 30 ºC for 1 h. Control reactions included only 150 µl soya tryptone broth. Following incubation, 50 µl of each peptide/ECP mixture were transferred to 96 well micro-titer plates. Their activity against the easily manipulated *Escherichia coli* (ATCC25922) bacterium were tested by growth inhibition assay as described in section 2.2.5.

4.3 Results

4.3.1 Secretion of proteases on agar media

A. hydrophila was shown to produce protease as indicated by clear zones around colonies (Fig. 4.1A) while no protease activity was detected for *K. pneumoniae* (Fig. 4.1B).

4.3.2 Protease activity of ECPs

The presence of protease activity in the ECPs of *A. hydrophila* was dependent on the incubation time of the cultures (Fig. 4.2A). No activity was detected in the ECPs from 0 h and 8 h cultures, while the activity of the ECPs from the 16 h, 24 h and 36 h cultures increased with incubation time of the cultures (Fig. 4.2A). The activity was lost upon treatment with protease inhibitors. No protease activity was detected in the ECPs of *K. pneumoniae* (Fig. 4.2B). Heat treated ECPs of both bacterial cultures did not show protease activity (Fig. 4.2).

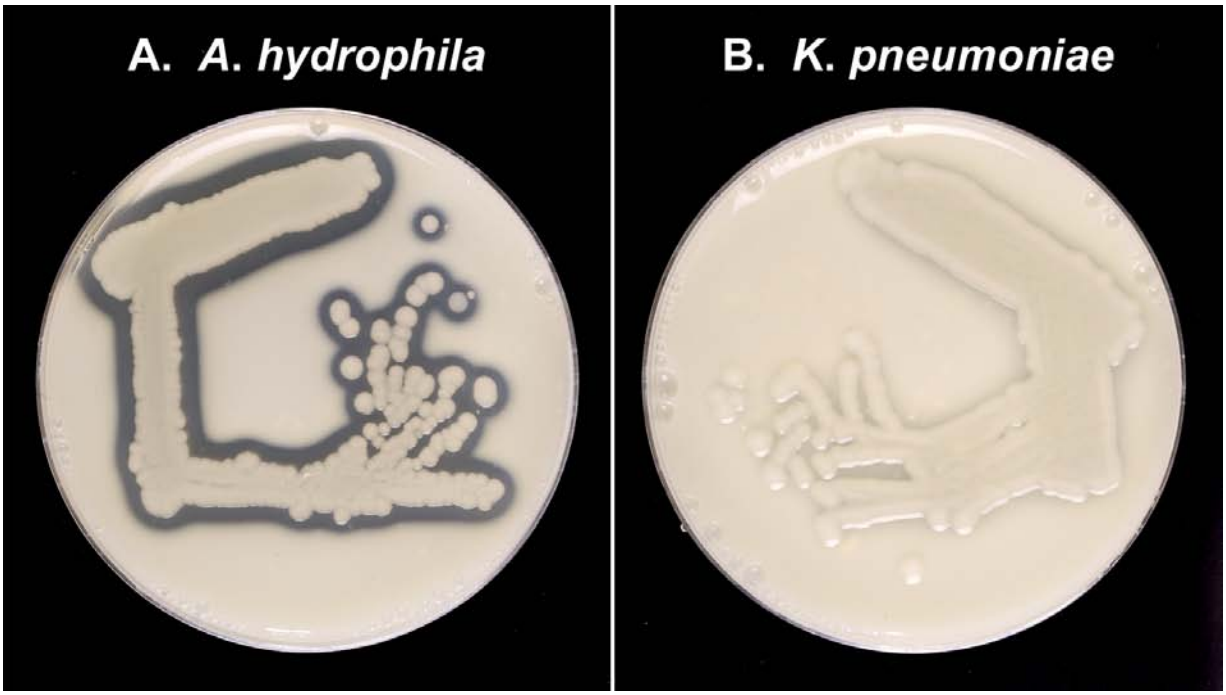

Fig. 4.1 Analysis of protease activity of the ECPs of *A. hydrophila* (A) and *K. pneumoniae* (B) on skim milk-agar media

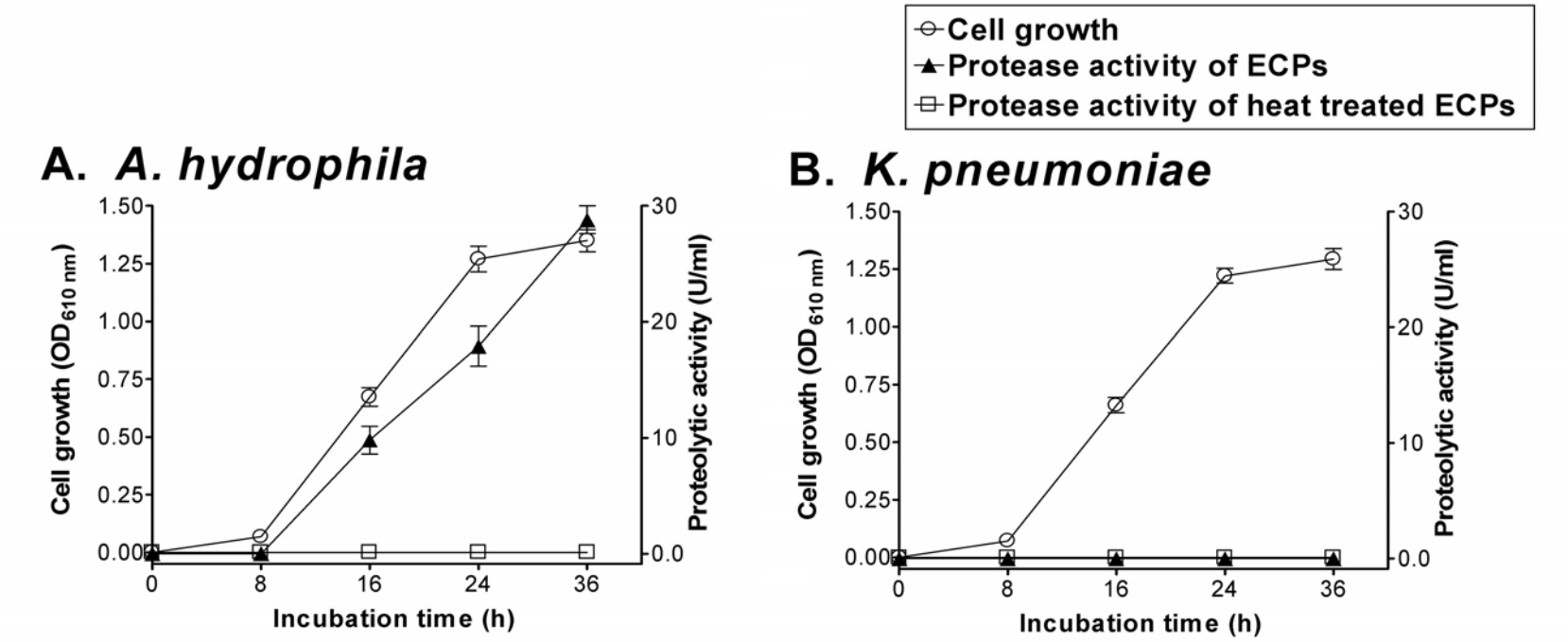

Fig. 4.2 Cell growth and protease activity of ECPs of *A. hydrophila* (A) and *K. pneumoniae* (B) cultures at different incubation time.

4.3.3 ECP mediated inhibition of activity of skin peptides

A. hydrophila ECPs were shown to inhibit the single peptides and peptide mixtures from *X. laevis*, *L. aurea* and *L. raniformis* (Figs. 4.3 and 4.4). The activity was dependent upon the incubation time of *A. hydrophila* in culture. Activity was only found in cultures grown for 16 h and longer (Figs. 4.3 and 4.4). It is interesting to note that *A. hydrophila* ECPs did not affect the activity of polymyxin B (Fig. 4.4); however, polymyxin B has disulfide bonds in its structure which may confer greater stability to proteases as shown by Paulus and Gray (1964). The ECPs of *K. pneumoniae* which were not proteolytic in nature had no effect on the frog peptides (Figs. 4.5 and 4.6). Heat treatment of ECPs abolished any inhibitory effects on the skin peptides (Figs. 4.3–4.6).

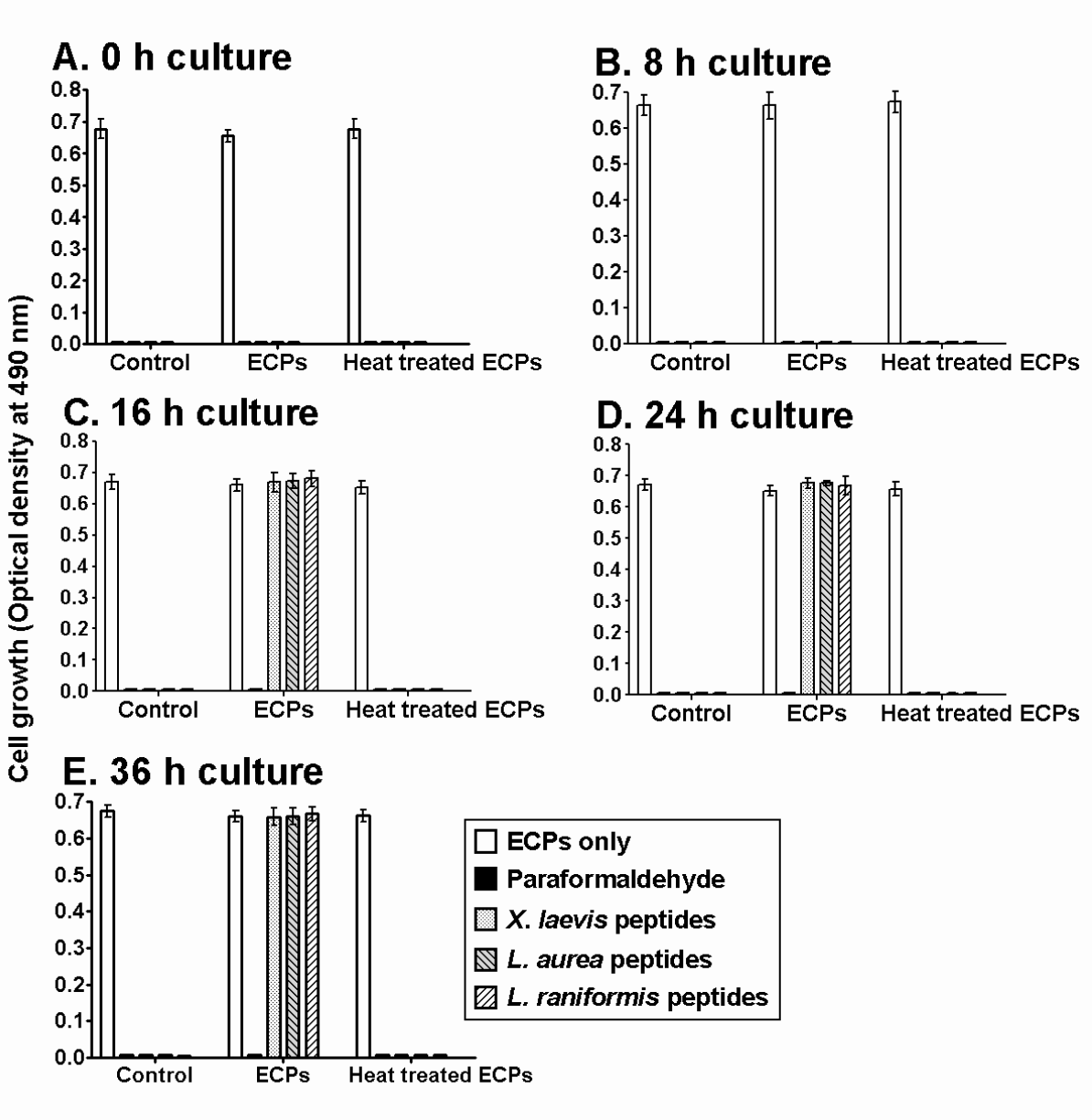

Fig. 4.3 Activity of peptide mixtures of *X. laevis, L. aurea* and *L. raniformis* against *E. coli* (ATCC25922) after treatment of the peptides with ECPs from *A. hydrophila* cultures grown for 0 h (A), 8 h (B), 16 h (C), 24 h (D) and 36 h (E).

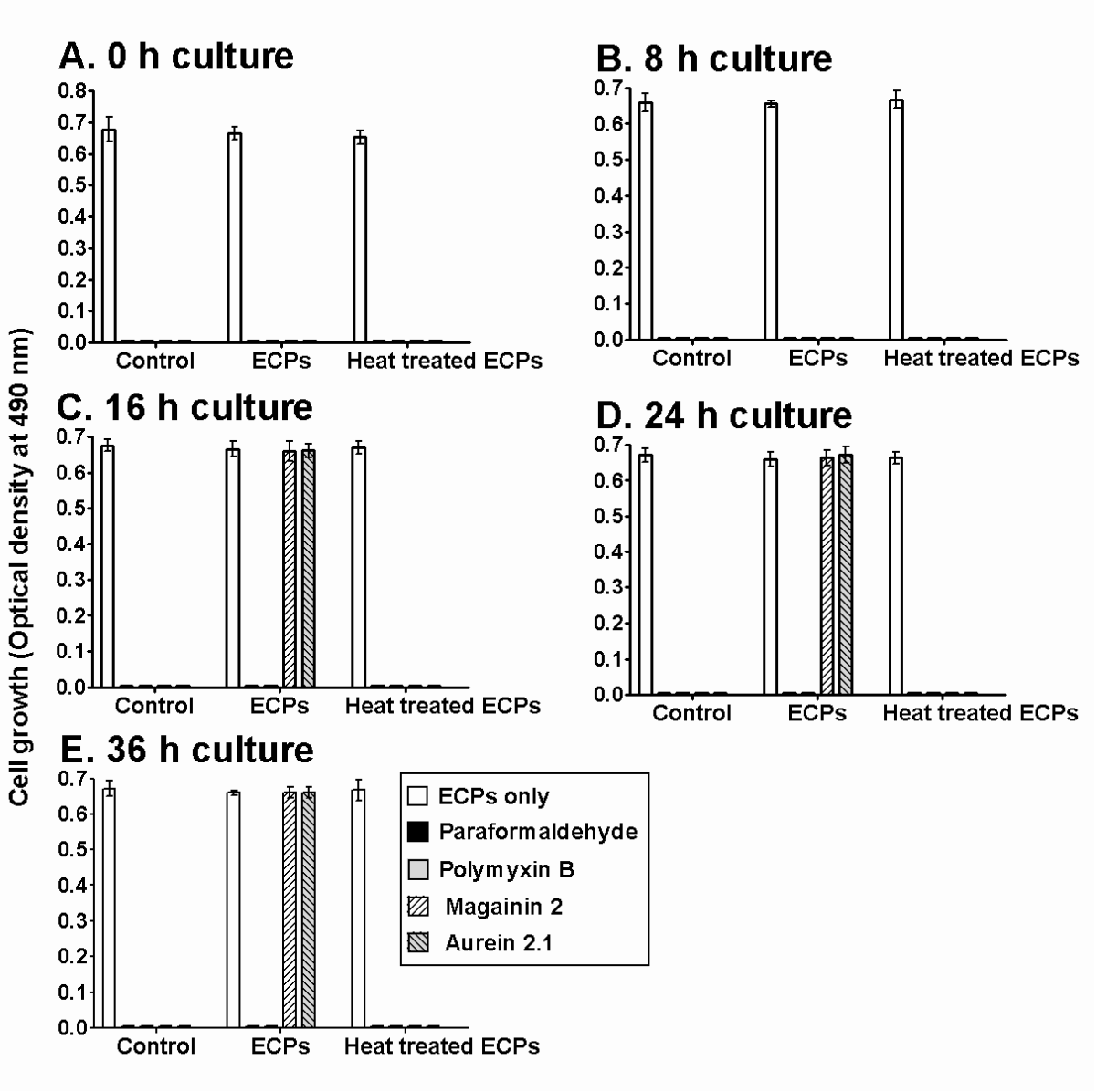

Fig. 4.4 Activity of polymyxin B, magainin 2 and aurein 2.1 against *E. coli* (ATCC25922) after treatment of the peptides with ECPs from *A. hydrophila* cultures grown for 0 h (A), 8 h (B), 16 h (C), 24 h (D) and 36 h (E).

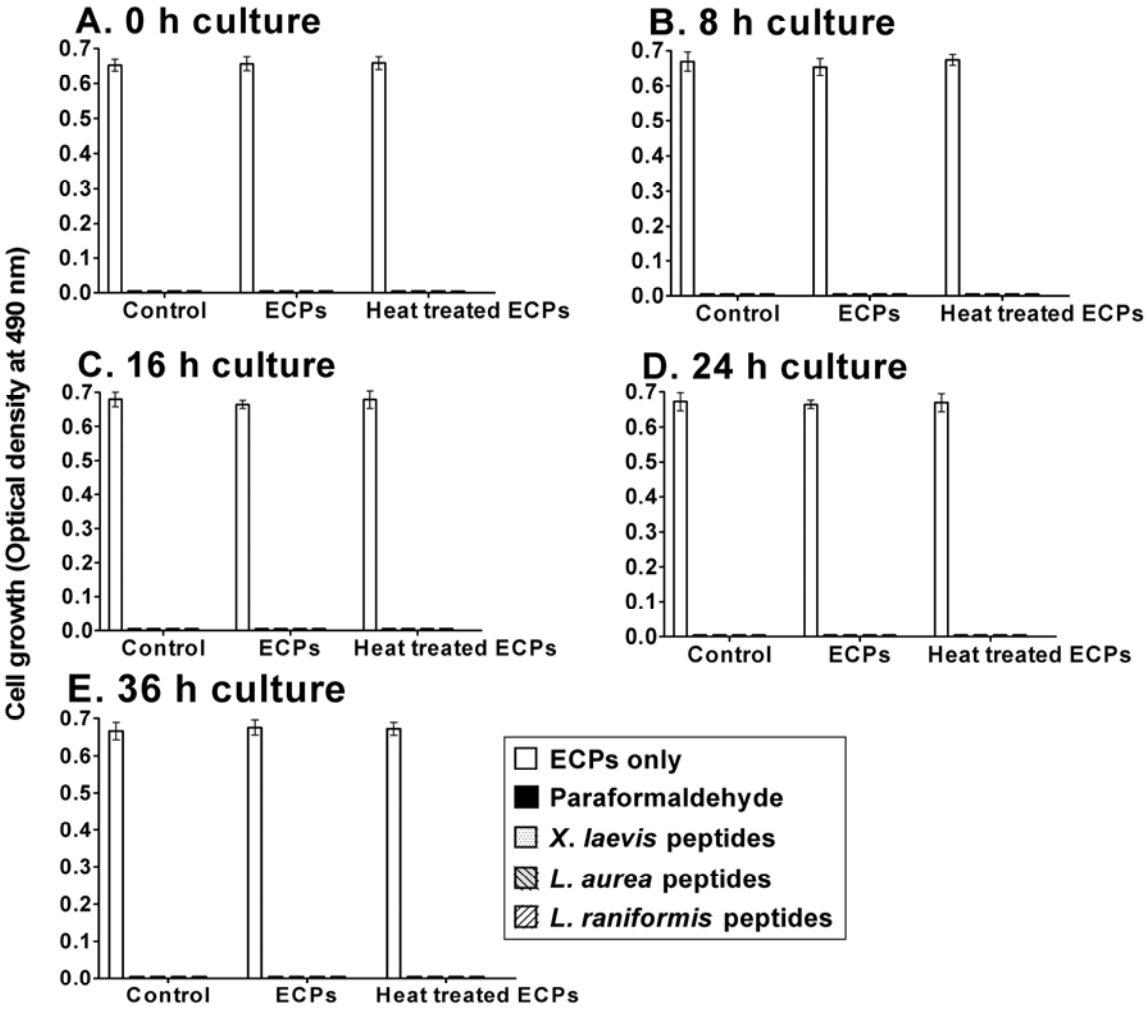

Fig. 4.5 Activity of peptide mixtures of *X. laevis, L. aurea* and *L. raniformis* against *E. coli* (ATCC25922) after treatment of the peptides with ECPs from *K. pneumoniae* cultures grown for 0 h (A), 8 h (B), 16 h (C), 24 h (D) and 36 h (E).

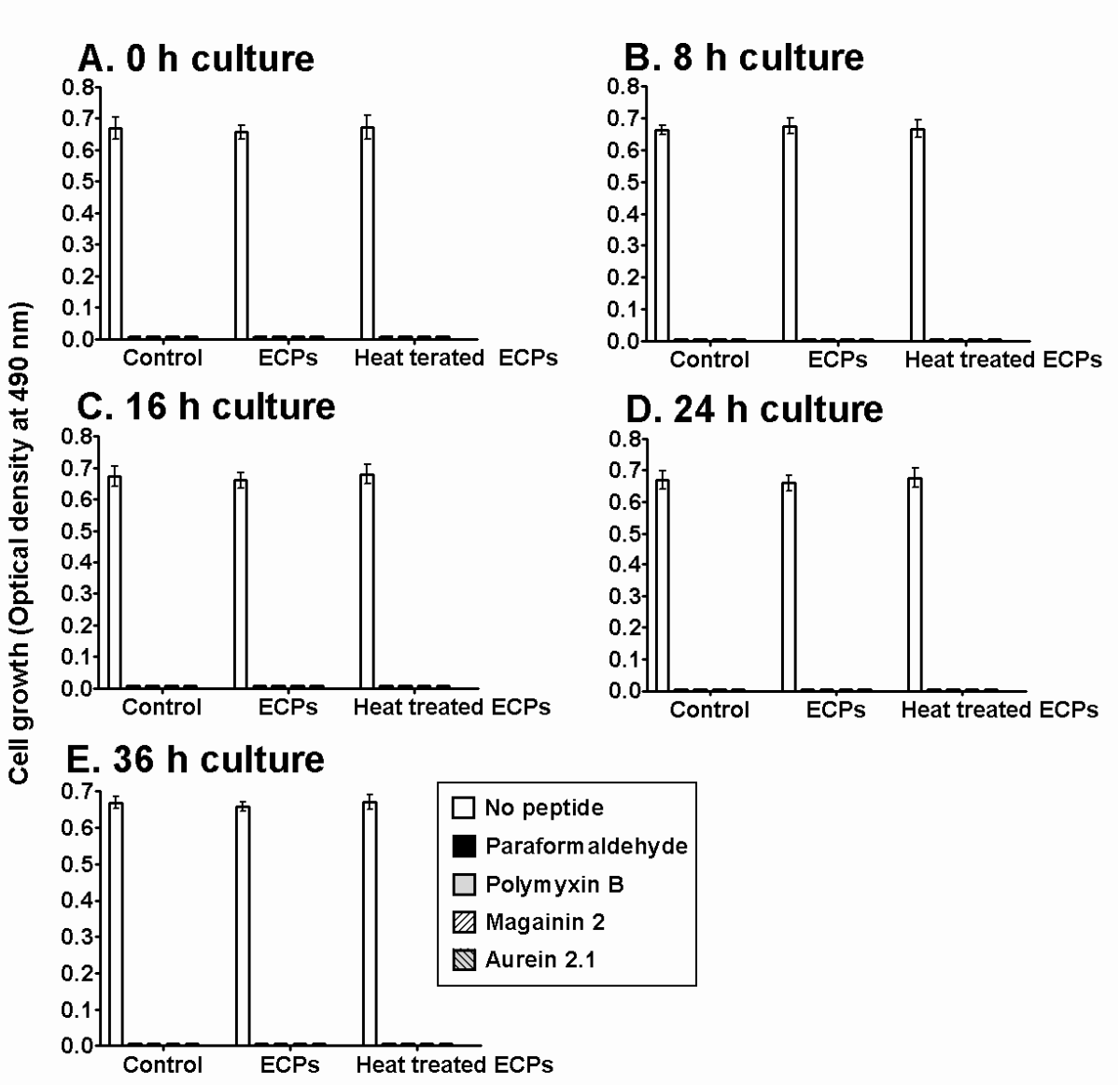

Fig. 4.6 Activity of polymyxin B, magainin 2 and aurein 2.1 against *E. coli* (ATCC25922) after treatment of the peptides with ECPs from *K. pneumoniae* cultures grown for 0 h (A), 8 h (B), 16 h (C), 24 h (D) and 36 h (E).

4.4 Discussion

The ECPs of *A. hydrophila*, which are proteolytic in nature, appear to be responsible for the resistance of this bacterium to the skin peptides of three frog species *X. laevis*, *L. aurea* and *L. raniformis*. Significantly, the ECPs of *A. hydrophila*, a pathogen resistant to

peptide mixtures and single peptides of all three frog species, completely inhibited the activity against *E. coli* (ATCC25922) of the peptides of these frog species (Figs. 4.3 and 4.4), The ECPs were inhibited by heat treatment (Fig. 4.2A). Moreover, the ECPs of *K. pneumoniae,* a pathogen susceptible to peptide mixtures and single peptides of all three species, did not have protease activity and did not inhibit the activity of the frog skin peptides against *E. coli* (ATCC25922) of peptide mixtures or single peptides of all three species (Figs. 4.1, 4.2B, 4.5, 4.6). The virulence of *A. hydrophila* would, therefore, appear to be associated with the production of proteases and this is consistent with the hypothesis of Pearson (1998) that *A. hydrophila* could have mechanisms to evade destruction by immune defences and thereby ensure a sufficient number of bacterial cells required for tissue invasion in the initial phase of disease.

It is also possible proteases provide better acquisition of nutrients in infected tissues as suggested by the degradation of skim milk proteins (Fig. 4.1A) and promote bacterial invasion of tissues. Furthermore, it is possible that after bacterial entry into blood and lymph, proteases could inhibit innate immune defence mechanisms involving macrophages and natural killer cells by degrading cytokines. These proteins are required for activation of these cells, and thereby ensure systematic dissemination of pathogen.

A. hydrophila is commonly found in diseased animals of many different frog species (Hird *et al*., 1981, Taylor *et al*., 2001), which might suggest that production of proteases evolved initially as a trait required for adaptation to the skin of their host. This suggestion is supported by the fact that protease-mediated resistance to skin peptides could enable *A.*

hydrophila to survive on the skin. These proteases might be specific to host peptides as the inhibition of the activity of polymyxin B, a non-frog antimicrobial peptide, was not seen (Fig. 4.4).

Although proteases of *A. hydrophila* could be associated with resistance of the bacterium to skin peptides (Figs. 4.3 and 4.4), other mechanisms may be also in place. Some animal and human isolates of *Aeromonas*, *Proteus* and *Serratia* spp. also have the ability to develop resistance to different kinds of antimicrobial peptides by modification of lipopolysaccharide (LPS) and protein components in their outer membranes (Storm *et al*., 1977, Smeianov *et al*., 2000, McCoy *et al*., 2001, Rosenfeld *et al*., 2006). Therefore future studies on resistance of pathogens to skin peptides should address this issue.

Although resistance mechanisms of opportunistic bacteria to skin peptides might be species specific, variations in these mechanisms might occur among different isolates. One direction of future research would be to compare resistance to skin peptides among different isolates and different species.

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Chapter 5: Bath Challenges with Bacterial Isolates from Wild Frogs

5.1. Introduction

Bacterial dermatosepticemia is a common disease of frogs. Its epizootic forms are associated with mass mortalities in wild populations (Nyman, 1986, Bradford, 1991) and significant losses in captive populations (Olson *et al*., 1992, Taylor *et al*., 1993, Green *et al*., 1999).

Different opportunistic Gram-negative bacterial species have been isolated from diseased frogs including *Aeromonas hydrophila, Chryseobacterium indologenes, Chryseobacterium meningosepticum, Citrobacter freundii, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa* and *Serratia liquefaciens* (Hunsaker and Potter, 1960, Glorioso *et al*., 1974, Taylor *et al*., 2001, Mauel *et al*., 2002). These bacterial species can be found amongst the microbiota of skin and guts of healthy frogs but are also known to cause disease (Hird *et el*., 1981, Pearson, 1998, Taylor, 2001, Hayes *et al*., 2006). Disease development depends on their virulence and environmental factors (Glorioso *et al*., 1974, Rigney *et al*. 1978, Pearson, 1998, Pearson *et al*., 2000).

The virulence of bacterial pathogens is usually evaluated using pathogenicity models which aim to reproduce disease *in vivo* by challenging the selected host with bacterial isolates. These models enable disease to be monitored and are essential in the comparison of virulence of different bacterial isolates (Pearson, 1998). In addition, they can be used to evaluate efficacy of prophylactic and chemotherapeutic treatments (Janda, 1991), to

investigate the relationship between environmental factors and bacteria in the aetiology of disease (Janda, 1991) and to assess the function of immune defences (Woodhams *et al*., 2007).

Two modes of bacterial challenge are used in frog pathogenicity models: injection and bath challenge. Injection of pathogens is widely used for analyses of virulence of human and animal bacterial isolates but its main flaw is that bacteria are introduced in a manner that does not adequately reflect the natural mode of infection (Wise *et al*., 1993, Pearson, 1998). Feeding frogs with various bacterial pathogens might resemble a more natural mode of infection but it does not result in disease (Glorioso *et al*., 1974). Under experimental conditions it is found that immersion in a bacterial pathogen suspension results in disease and appears to more closely simulate the natural mode of infection because none of the natural immune defence barriers are by-passed (Pearson, 1998).

Four requirements for proof of virulence of an organism were presented in 1883 by the German bacteriologist, Robert Koch. His postulates state that an organism is an causative agent of disease if:

1. It is present in all cases of disease.
2. It must be isolated from a diseased organism and grown in pure culture.
3. It causes disease when introduced into a healthy organism.
4. It must be re-isolated from the inoculated, diseased experimental host and identified as being identical to the original specific causative agent.

This framework is relatively rigid for demonstrating that a bacterium is a potential pathogen. The influences of environmental factors are not considered, although they are important in development of bacterial disease of frogs. Despite such limitation, the postulates are still a standard by which association of bacteria with disease could be established (Thrushfield, 1995).

The aims of this study were to assess the virulence of three bacterial isolates collected from wild *Litoria ewingii*: *A. hydrophila, K. pneumoniae* and *P. mirabilis*. Isolates were used to challenge *L. ewingii* and their ability to fulfill the third and fourth Koch's postulates were investigated.

5.2 Material and Methods

5.2.1 Frogs

Adult *L. ewingii* (2.0-2.5 g) were collected in Oxford Forest in January 2008. They were maintained in natural conditions in a vivarium. One month before experimentation they were transferred to Sistema 7L polypropylene box tanks with a snap lid (350 x 200 x 100 mm) and maintained as described in section 2.2.7.

5.2.2 Bacterial cultures

The isolates of *A. hydrophila, K. pneumoniae* and *P. mirabilis* were as described in section 2.2.4.

5.2.3 Calibration curve for bacterial cultures

A colony from overnight growth on tryptone soya agar was used to inoculate 10 ml of tryptone soya broth (TSB). Bacterial cells were harvested by centrifugation at 4,000 g at 4ºC for 15 min and resuspended in sterile saline (0.85% NaCl). The optical density (at 610 nm) of bacterial suspension was adjusted with sterile saline to give an optical density of 1.2, 1.0, 0.8, 0.6, 0.4, 0.2 and 0.1. At each optical density a sample was taken to estimate number of colony forming units (cfu) by overnight incubation on tryptone soya agar. The number of cfu was plotted against optical density to give a calibration curve for the number of cfu (standard curves are shown in Appendix J). The regression equation obtained from the calibration curve was used to estimate the number of cfu in the broth cultures.

5.2.4 Preparation of bacterial challenge inoculum

Cells from an overnight bacterial TSB culture were harvested by centrifugation at 4,000 g at 4ºC for 15 min and resuspended in sterile saline. The optical density of bacterial suspensions was measured and number of cfu/1 ml of suspension was estimated using the calibration curves and bacterial suspensions diluted with sterile saline to give a final concentration of 2×10^8 cfu/ml suspension which was used as the inoculum. Sterile saline was used inplace of inoculum in control treatments.

5.2.5 Bacterial baths

Two successive short baths were performed instead of an overnight bath that could disturb osmoregulation of arboreal species like *L. ewingii*. Each animal was put into 300

ml of pond water with bacteria at a concentration of 2 x 10^7 of cfu/1ml of pond water in 500 ml tissue culture jars and monitored during a 40 min bath. The lid of the jars had drilled ventilation holes. Between two baths the animal was put to rest in 7 L empty polypropylene tanks for 30 min. After the trial, the animals were returned to their living containers and their health was monitored for a period of three weeks.

5.2.6 Euthanasia of animals

Diseased animals were euthanised by a humane euthanising agent (3% aqueous solution of chloral hydrate) to prevent suffering. Samples of liver, heart and spleen were taken for culturing bacteria as described in section 2.2.4. At the end of experiment, all animals were euthanised and samples of internal organs also taken for bacterial culturing.

5.2.7 Identification of bacteria

Bacteria from internal organs were identified using the API 20E identification kit (Biomérieux, Marcy-L'etoile, France) containing 23 conventional biochemical tests in miniaturised forms. It consists of microtubes with dehydrated media which are reconstituted with suspension of bacterial cultures. During incubation, metabolism produces colour changes that are either direct or revealed by the addition of reagent. The strips were inoculated according to the manufacturer's instructions. In brief:

- An API strip was placed in a moist incubation tray.
- A colony from the overnight an overnight soya tryptone agar culture was resuspended in 5 ml of sterile saline.

- The suspension was aliqouted into the microtube. Those tests requiring anaerobic conditions were covered with mineral oil.
- The strip was covered and incubated overnight at 30°C.
- The strip was examined and direct reactions were recorded. Additional reagents were added when required.
- The results were read by referring to the manufacturer's interpretation table in API 20 E manual.
- Four supplementary tests were performed aside the strip: analysis of motility, growth on MacConkey agar, oxidation of glucose and fermentation of glucose. Information on procedures provided in appendices K–M.
- The identification of bacteria was carried out with biochemical profiles obtained by API 20 E tests and four supplementary tests using Apiweb software (Biomérieux, Marcy-L'etoile, France). Information on biochemical profiles provided in appendix N.

5.3 Results

Morbidity and mortality were recorded only in *K. pneumoniae* challenge. Two animals developed disease two days after exposure and died seven days after exposure (Table 5). They had gross clinical signs of disease including haemorrhages, ulcers and redness of the ventral skin area (Fig. 5). The challenge bacterium was isolated as a pure culture from their heart, spleen and liver. The remaining animals in the *K. pneumoniae* challenge and all of the animals in the two other challenges and control group remained healthy until the end of the experiment (Table 5). Samples from their internal organs were negative for bacterial growth.

Table 5 Mortality of *L*. ewingii following bath challenges with 2 x 10^7cfu/1 ml of pond water of *A. hydrophila, K. pneumoniae* and *P. mirabilis*.

Treatment	1 day	2 days	3 days	4 days	5 days	6 days	7 days	Final mortality (%)
A. *hydrophila* (N = 5)	0	0	0	0	0	0	0	0
K. *pneumoniae* (N = 5)	0	0	0	0	0	0	2	40
P. *mirabilis* (N = 5)	0	0	0	0	0	0	0	0
Control (N = 5)	0	0	0	0	0	0	0	0

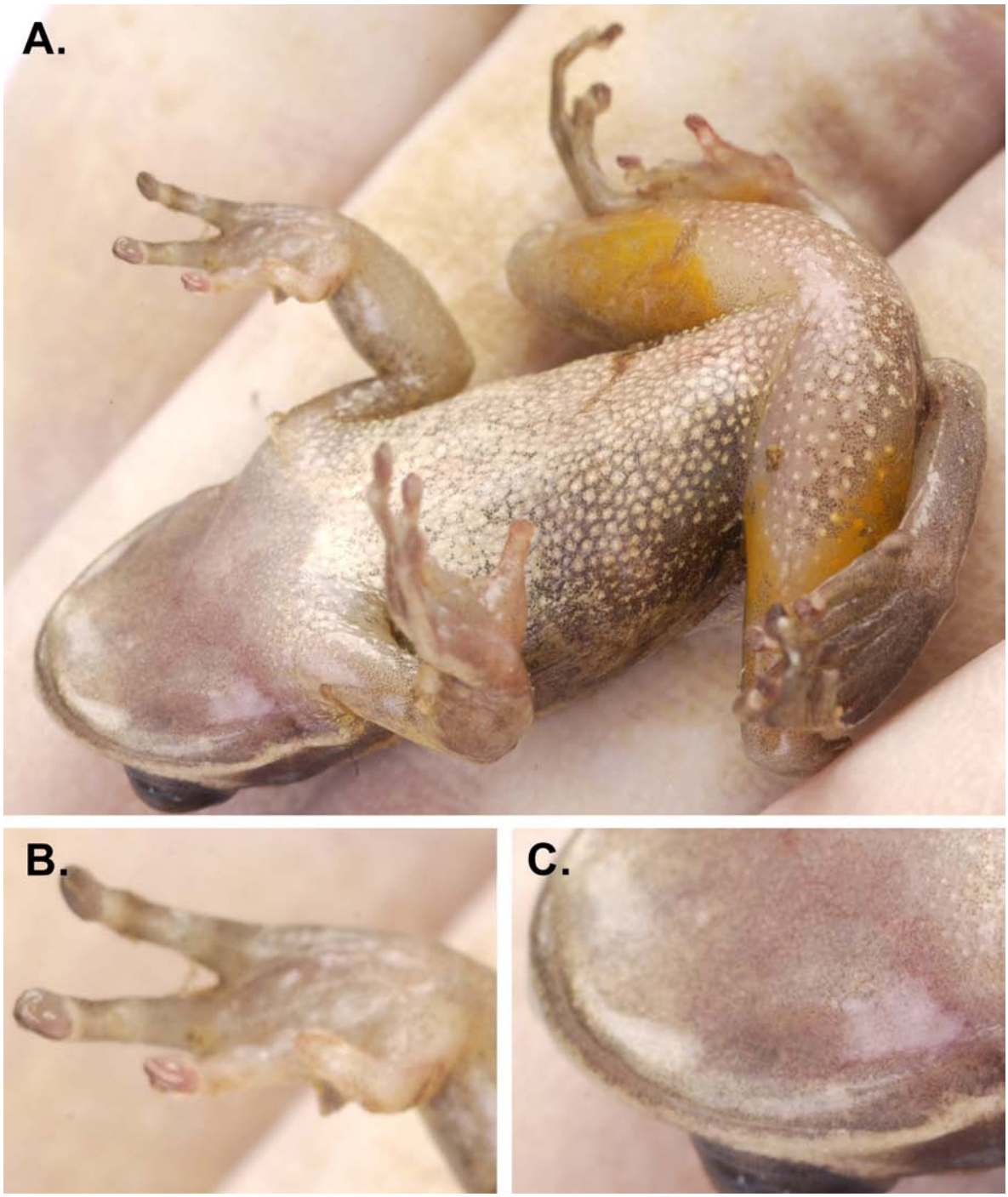

Fig. 5 Gross clinical signs of *L. ewingii* infected with *K. pneumoniae*: body posture (A), skin ulcer (B) and prominent redness of ventral skin (C).

5.4 Discussion

The results from this study demonstrated the virulence of *K. pneumoniae* towards *L. ewingii*. *K. pneumoniae* was isolated from diseased L. ewingii, grown in pure culture, induced disease in healthy individuals and was re-isolated to fulfill three of four Koch's postulates for disease caused by this microorganism. The animals developed septicemic disease and the bacterium was re-isolated from the diseased animals (Fig. 5; Table 5).

The septicemic disease demonstrated by the infected frogs was slow progressing confirming the results of Pearson (1998) who found that the bacterial baths of frogs with *Aeromonas* sp. at doses of 10^6 cfu caused slowly progressing septicemic disease while *Aeromonas* injection caused a rapid septicemic disease and death within twenty-four hours. Slow progression of disease in the bath challenge might be due to the time taken for developing skin infections preceding systematic bacterial dissemination (Pearson, 1998).

K. pneumoniae is not a frog specific pathogen although its virulence was demonstrated for *L. ewingii* (Fig. 5; Table 5). This bacterium has been associated with disease outbreaks in humans and different animals (Enurah *et al*., 1988, Brisse and Van Duijkeren, 2005, Munoz *et al*., 2006, Castinel *et al*., 2007). Different mammalian species have been described to become infected by contaminated water or soil as the bacterium can survive for a long time outside of its host (Enurah *et al*., 1988, Munoz *et al*., 2006, Castinel *et al*., 2007). The ability of these animals to resist infection depends on different mechanisms of the innate immune defences as the adaptive immune defences cannot provide a rapid efficient response to the high number of invading pathogens (Yoshida *et al*., 2001, Cortès, *et al*., 2002). The observation that *L. ewingii*,

the only introduced *Litoria* species that does not have the active antimicrobial peptide of the skin innate immunity (described in chapter two), is susceptible to disease both in the wild (Schadich unpubl. data) and in bath challenges (Fig. 5; Table 5) supports a role for the innate immune defences of frogs in protection from *K. pneumoniae*.

Virulence of the isolates of *A. hydrophila* and *P. mirabilis* that did not cause disease of *L. ewingii* cannot be ruled out at this stage since bath challenges without skin scarification might underestimate it. Somsiri *et al.* (1997) reported that skin scarification is required for demonstration of virulence of some frog bacterial isolates. In addition, the virulence of isolates was tested only at a bacterial dose of 2×10^7 cfu/ 1 ml of pond of water and different bacterial doses might give different results. Thus a more sensitive bath challenge model with skin scarification of tested frogs and different doses of challenge bacteria would be required to rule out the possibility that these isolates are in fact potentially virulent.

Environmental stressors have profound and diverse effects on the immune defences of frogs. Their inhibitory effects on resistance to disease have been demonstrated in many different studies (Rigney *et al.* 1978, Carey and Bryant, 1995, Taylor *et al.* 2001, Hayes *et al.* 2006). Somsiri *et al.* (1997) found that low temperature was one of the most significant stressors that increased morbidity and mortality of frogs in bath challenges with *A. hydrophila*. One direction of future studies should include an analysis of the role of low temperature in disease resistance of frogs.

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Chapter 6: Effects of the Pesticide Carbaryl and the Bacterial Pathogen *Klebsiella pneumoniae* on Production of Skin Peptides

6.1 Introduction

Among different pollutants, pesticides have most often been cited as having a primary role in amphibian decline (Davidson *et al*., 2004, Davidson and Knapp, 2007). The decline of four *Rana* species: *R. boylii*, *R. cascadae*, *R. draytonii* and *R. muscosa* have been observed in a region with intensive pesticide drifts (Drost and Fellers, 1996, Davidson *et al*., 2004, Davidson and Knapp, 2007). The documented levels of various pesticides in these regions of decline are generally of several orders of magnitude below the lethal concentrations as determined in laboratory studies (Davidson *et al*., 2004). However, sub-lethal concentrations of different pesticides including two common pesticides: the herbicide atrazine and the insecticide carbaryl can affect different physiological processes of frogs including their immune defences. In *Rana pipiens*, exposure to atrazine caused suppression of both adaptive and innate immune defences as shown by decreased antibody production (Gilbertson *et al*., 2003) and phagocytic activity of white blood cells respectively (Brodkin *et al*., 2007). In *R. boylii*, exposure to carbaryl caused reduced production of the antimicrobial peptides of the skin immune defences (Davidson *et al*., 2007). Thus, if pesticides are implicated in amphibian decline, it is probably due to synergistic effects with other factors such as disease.

Bacterial dermatosepticemia, a bacterial infectious disease, has been observed to affect populations of the introduced *Litoria* species *L. ewingii* in Oxford Forest, Canterbury

(Schadich unpubl. data). Bacteria isolated from diseased frogs, *K. pneumoniae,* were able to fulfill Koch' postulates (described in Chapter 5) and this suggests it could also pose a risk to another introduced *Litoria* species, *L. raniformis,* which is also found in this region. Resistance to disease could depend on effective immune defences such as the antimicrobial peptides of the skin innate immune defences (Simmaco *et al*., 1998, Rollins-Smith *et al*., 2005, Woodhams *et al*., 2006, Woodhams *et al*., 2007). Although an extensive analysis showed that *L. raniformis* had more efficient skin peptides than *L. ewingii* (described in Chapter 2), it is possible that, in the regions of the South Island of New Zealand affected by pesticide drift, exposure to pesticides could inhibit skin peptides and thereby increase susceptibility of *L. raniformis* to disease (Stevens, 1993, Holland and Rahman, 1999, Davidson and Knapp, 2007).

The aim of this study was to assess the possible effects of the pesticide carbaryl and the bacterium *K. pneumoniae* on production and activity of skin peptides, and disease development of *L. raniformis*.

6.2 Materials and methods

6.2.1 Frogs

Adult *L. raniformis* (26-33 g) were purchased from the commercial supplier Sweet Koura Enterprises, Alexandra in December 2007. The animals were maintained in natural conditions in a vivarium. One month before experiment they were transferred in Sistema 7L polypropylene box tanks with a snap lid (350 x 200 x 100 mm) and maintained as described in Section 2.2.7.

6.2.2 Bacterial culture

The isolate of *K. pneumonia*e from wild *L. ewingii* was as described in section 2.2.4.

6.2.3 Carbaryl

Carbaryl (98% purity) was purchased from Kiwi Care Ltd, Christchurch.

6.2.4 Exposure to carbaryl and *K. pneumoniae*

L. raniformis frogs were assigned to four different groups of twenty labelled according to assigned treatment: carbaryl, *K. pneumoniae*, carbaryl and *K. pneumoniae* and control. On the first day of the trial, carabaryl and carbaryl and *K. pneumoniae* groups were exposed to carbaryl. Each animal was placed into a container with 3.5 L of pond water with carbaryl at the ecologically relevant concentration of 0.48 mg/L in a 7L tanks that allowed the animals to swim or to stand with its head above the surface water and monitored during a 16 h exposure. The lids of the containers had drilled ventilation holes. After exposure, frogs were returned to the container in which they were normally kept. On the second day, *K. pneumoniae* and carbaryl and *K. pneumoniae* groups were exposed to *K. pneumoniae* as described by Pearson (1998). Each animal was placed into a container with 3.5 L of pond water with *K. pneumoniae* at a concentration of 2 x 10^7colony forming units for 16 h. The control group was exposed only to pond water both on the first and second days. After exposure, the animals were returned to their living tanks and monitored for survival for the period of three weeks. Skin peptides were collected twenty-four days before and two days after exposure to carbaryl or one day after exposure to *K. pneumoniae*. In order to exclude the possible effects of peptide collection on survival, four additional groups were exposed to carbaryl, *K. pneumoniae*, carbaryl and *K. pneumoniae* and pond water respectively but without collection of skin peptides.

Procedures for inducing, collecting and purifying skin peptides are described in Section 2.2.1. The quantity of skin peptides recovered and their activity against the isolate of *K. pneumoniae* were measured for each animal as described in Sections 2.2.1, 2.2.2, 2.2.5 and 2.2.6 respectively. Differences between the two groups of animals in total recovered peptides, MIC, MIC equivalents per gbm and surface area were tested by the nonparametric Kruskal-Wallis rank and Mann-Whitney U-tests.

6.2.5 LC-MS analyses of skin peptides profiles of *L. raniformis* exposed to carbaryl and *K. pneumoniae*

Liquid chromatography mass spectrometry (LC-MS) analyses of the samples of the natural peptide mixtures were carried out by the same procedure as described in Section 2.2.3.

6.3 Results

6.3.1 Effects of carbaryl and *K. pneumonia* on survival

Morbidity and mortality was not observed either in any individual from treated or control groups of *L. raniformis*.

6.3.2 Effects of carbaryl and *K. pneumoniae* on skin peptide defences

Before exposures of *L. raniformis* to *K. pneumoniae*, carbaryl and the combination of the two, no difference was found between the future exposed and control animals with respect to the following variables: the average total amount of recovered peptide (Kruskal-Wallis rank test, $X^2_3 = 0.80$, $P = 0.850$), the MIC of peptide mixtures against *K. pneumoniae* (Kruskal-Wallis rank test, $X^2_3 = 0.64$, $P = 0.887$), MIC equivalents per gbm (Kruskal-Wallis rank test, $X^2_3 =$

1.39, P = 0.707) and MIC equivalents per cm^2 (Kruskal-Wallis rank test, $X^2_3 = 2.17$, P = 0.537) (Table 6.1). After exposure, the total amount of recovered peptides and MIC equivalents per gbm and cm^2 of the exposed animals depended on the exposure. Animals exposed to *K. pneumoniae* had a significantly higher average of total recovered peptides (Mann-Whitney U-test, Z = –3.41, N = 40, P < 0.016), MIC equivalents per gbm (Mann-Whitney U-test, Z = –3.00, N = 40, P < 0.016) and MIC equivalents per cm^2 (Mann-Whitney U-test, Z = –2.89, N = 40, P < 0.016) than control animals (Figs. 6.1. and 6.2; Table 6.2). Animals exposed to carbaryl had a significantly lower average of total recovered peptides (Mann-Whitney U-test, Z = –5.36, N = 40, P < 0.016), MIC equivalents per gbm (Mann-Whitney U-test, Z = –5.08, N = 40, P < 0.016) and MIC equivalents per cm^2 (Mann-Whitney U-test, Z = –5.14, N = 40, P < 0.016) than control animals (Figs. 6.1 and 6.2; Table 6.2). Animals exposed to *K. pneumoniae* and carbaryl also had a significantly lower average of total recovered peptides (Mann-Whitney U-test, Z = –5.41, N = 40, P < 0.016), MIC equivalents per gbm (Mann-Whitney U-test, Z = –5.22, N = 40, P < 0.016) and MIC equivalents per cm^2 (Mann-Whitney U-test, Z = –5.22, N = 40, P < 0.016 than control animals (Figs. 6.1 and 6.2; Table 6.2). No difference was found between the exposed and control animals in average MIC (Kruskal-Wallis rank test, $X^2_3 = 0.44$, P = 0.933; Fig 6.3, Table 6.2). None of the peptide digests obtained by proteolysis of skin peptides inhibited bacterial growth.

Table 6.1 Production of skin peptides and peptide efficiency against *K. pneumoniae* of *L. raniformis* before exposure to carbaryl and *K. pneumoniae*, and carbaryl and *K. pneumoniae* simultaneously.

Treatment	No animals	Total peptides per 1g body weight ((µg/g) +/-SD)	MIC (µg/ml) +/- SD	MIC equivalents per 1g body weight (and +/-SD	MIC equivalents per cm^2 surface area (and +/-SD)
Pond water only (Control)	20	79.03 +/- 9.87	262.50 +/- 55.90	0.308 +/- 0.053	0.158 +/- 0.025
K. pneumoniae	20	80.88 +/- 6.95	256.25 +/- 63.80	0.330 +/- 0.076	0.169 +/- 0.041
Carbaryl	20	80.77 +/- 10.24	256.25 +/- 63.80	0.332 +/- 0.100	0.169 +/- 0.048
Carbaryl and *K. pneumoniae*	20	80.53 +/- 7.67	262.50 +/- 55.90	0.314 +/- 0.048	0.160 +/- 0.024

Table 6.2 Production of skin peptides and peptide efficiency against *K. pneumoniae* of *L. raniformis* exposed to carbaryl and *K. pneumoniae*, and carbaryl and *K. pneumoniae* simultaneously.

Treatment	N animals	Total peptides per 1g body weight ((µg/g) +/-SD)	MIC (µg/ml) +/- SD	MIC equivalents per 1g body weight (and +/-SD	MIC equivalents per cm^2 surface area (and +/-SD)
Pond water only (Control)	20	79.70 +/- 11.89	262.50 +/- 55.90	0.310 +/- 0.057	0.159 +/- 0.027
K. pneumoniae	20	95.02 +/- 12.77	268.75 +/- 83.85	0.379 +/- 0.107	0.193 +/- 0.053
Carbaryl	20	48.82 +/- 13.08	262.50 +/- 55.90	0.189 +/- 0.053	0.096 +/- 0.026
Carbaryl and *K. pneumoniae*	20	45.56 +/- 10.36	275.00 +/- 76.95	0.171 +/- 0.044	0.087 +/- 0.021

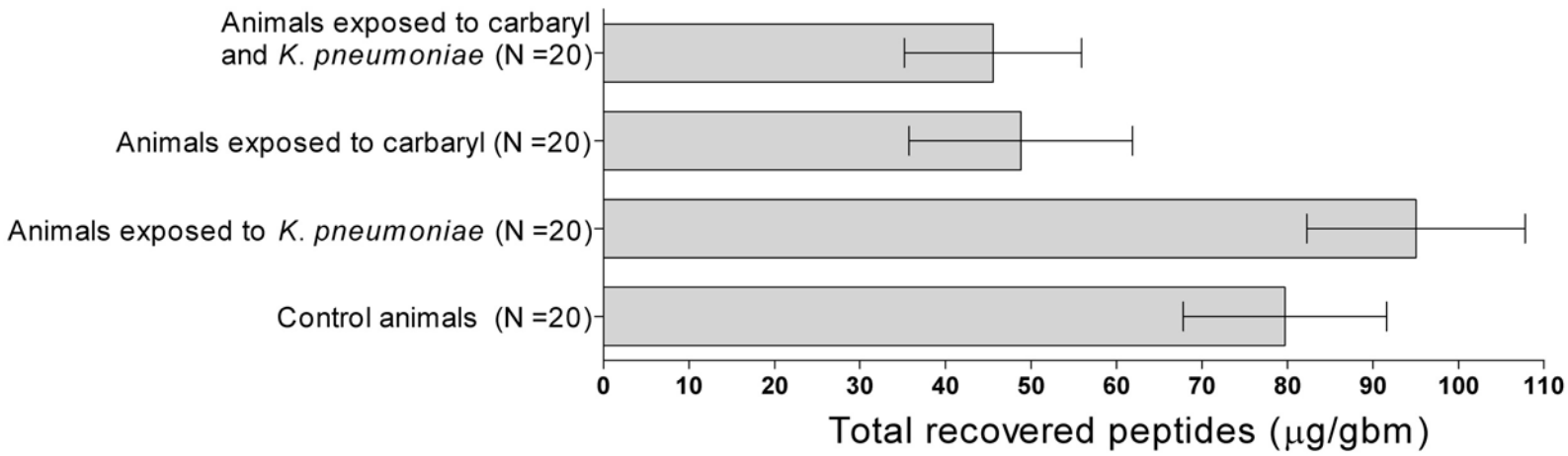

Fig. 6.1 Comparison of total recovered peptides of *L. raniformis* after exposure to pond water only (control), carbaryl, *K. pneumoniae* and carbaryl and *K. pneumoniae* simultaneously.

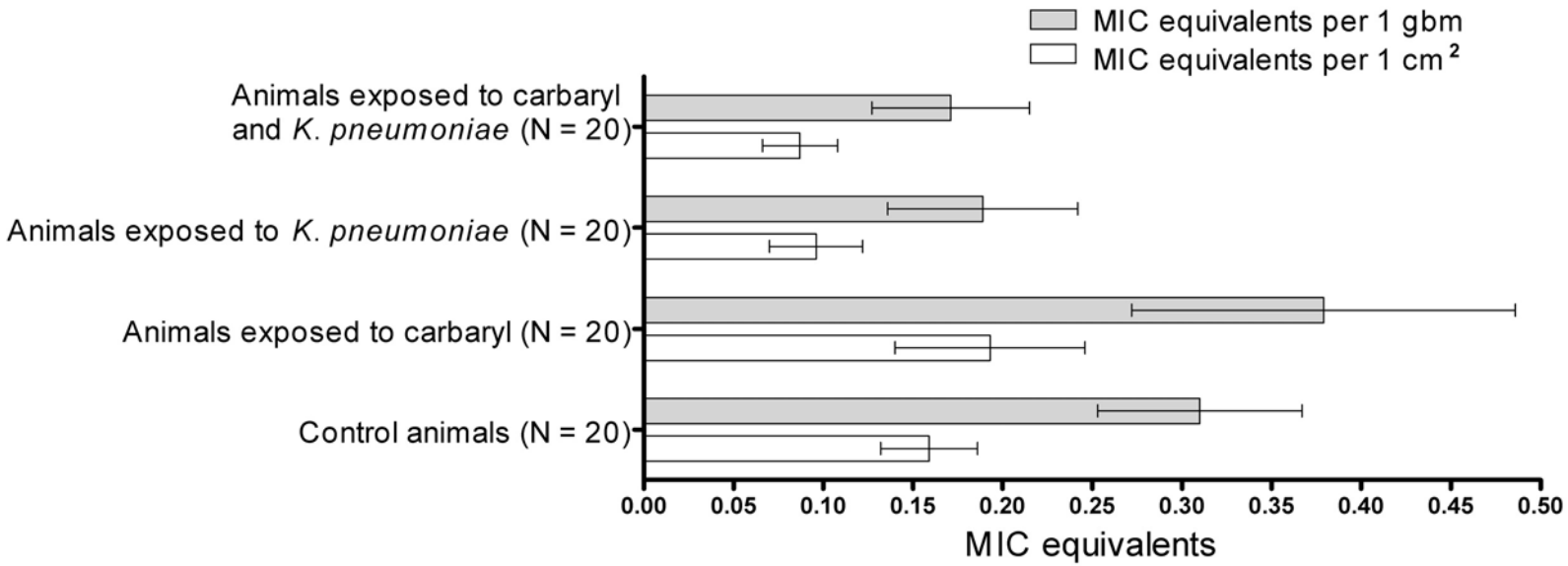

Fig. 6.2 Comparison of MIC equivalents of *L. raniformis* after exposure to pond water only (control), carbaryl, *K. pneumoniae* and carbaryl and *K. pneumoniae* simultaneously.

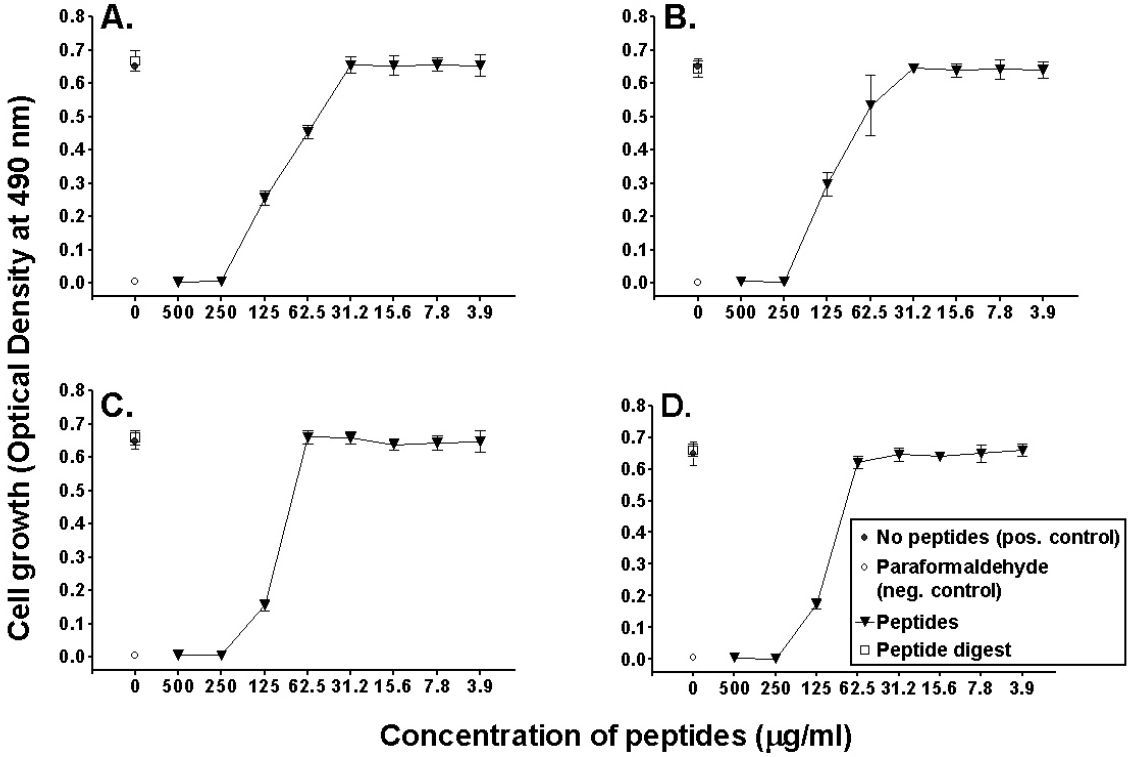

Fig. 6.3 An example of growth inhibition *K. pneumoniae* by skin peptides from *L. raniformis* exposed to pond water only (control) (A), *K. pneumoniae* (B), carbaryl (C) and carbaryl and *K. pneumoniae* (D).

6.3.3 Skin peptide profiles of *L. raniformis* after exposure to carbaryl and K. pneumoniae

Skin peptide profiles obtained by LC-MS were not markedly different among treated and control animals. The peptide mixtures from all had the peptides whose molecular masses (M_r) correspond to that of eight different aurein peptides: aurein 1.1 (M_r = 1444.48 Da), aurein 1.2 (M_r = 1479.27 Da), aurein 2.1 (M_r = 1614.53 Da), aurein 2.5 (M_r = 1646.49 Da), aurein 2.6 (M_r = 1627.65 Da), aurein 3.1 (M_r = 1737.59 Da), aurein 3.2 (M_r = 1767.67 Da) and aurein 5.2 (M_r = 2449.73 Da) (Fig. 6.4).

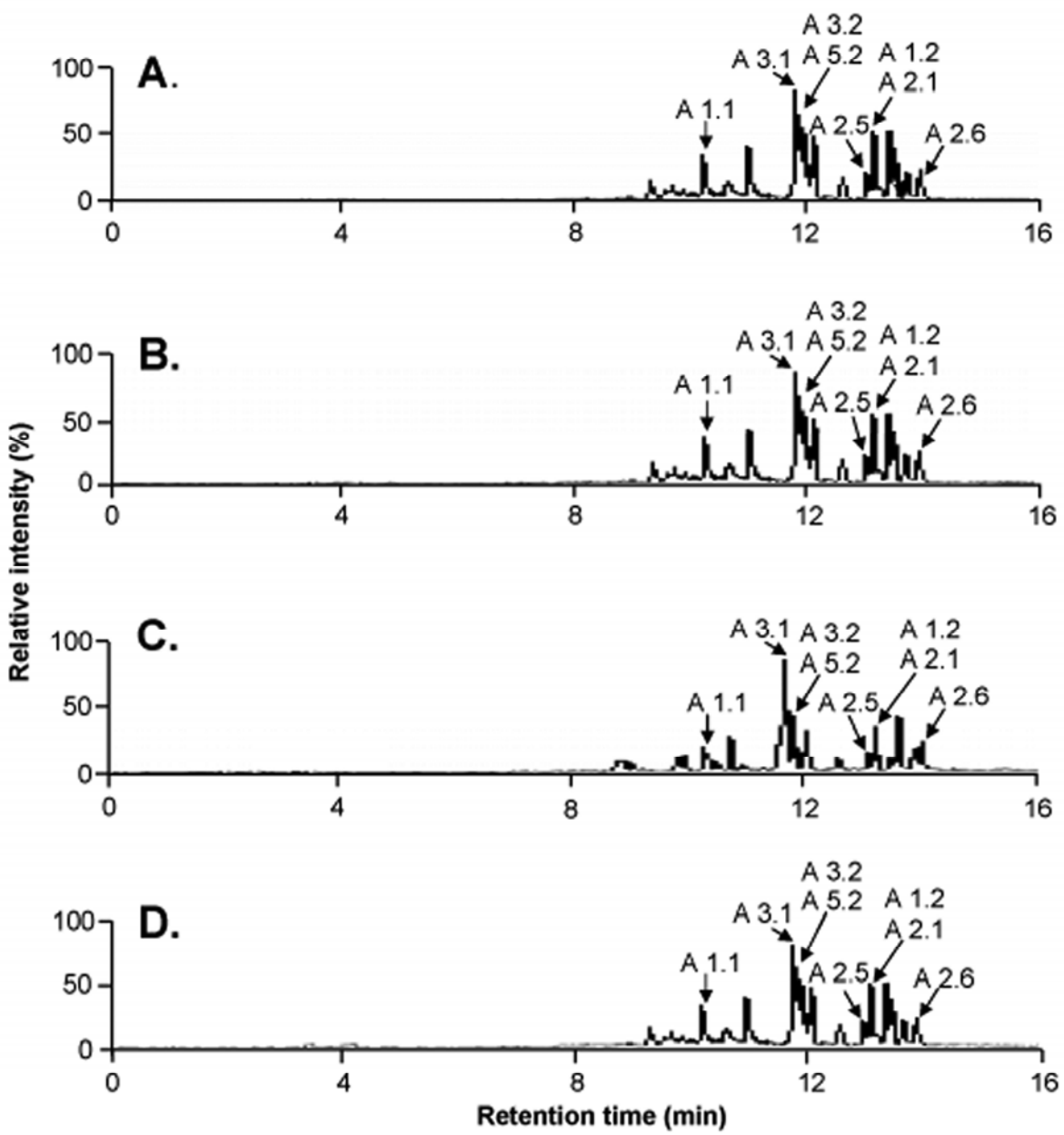

Fig. 6.4 LC-MS analyses of peptide mixtures from *L. raniformis*: exposed to pond water only (control) (A), *K. pneumoniae* (B), carbaryl (C) and carbaryl and *K. pneumoniae* (D).

6.4 Discussion

Based on the results presented in this chapter, *L. raniformi*s frogs might be well protected from bacterial disease induced mortality even in the presence of pesticide. Exposure to either pathogenic *K. pneumoniae* alone or in combination with carbaryl did not result in disease development. The ability of *L. raniformis* frogs to defend themselves from pathogenic disease may be attributable to the efficiency of their antimicrobial skin peptides. The natural mixture of skin peptides inhibited the growth of *K. pneumoniae in vitro* (Fig. 6.3). Although the total amount of recovered peptides was significantly decreased after exposure to carbaryl and *K. pneumoniae*, it was within the range that could provide significant protection (Figs. 6.1–6.3; Table 6.2). Thus, no interaction was observed between carbaryl and *K. pneumoniae* with respect to disease susceptibility.

6.4.1 Skin peptide response to *K. pneumoniae*

Skin peptide defences of *L. raniformis* to invading pathogens could be inducible as the total amount of recovered peptides was increased after exposure to *K. pneumoniae* (Fig. 6.1; Table 6.2). These results confirm the results of Simmaco *et al* (1998) who found that production of skin peptides of *Rana esculenta* increased after exposure to *A. hydrophila* through a response mechanism that includes the nuclear transcription factor (NF-κB factor) involved in activation of peptide synthesis in the granular glands (Miele *et al*., 1998, Simmaco *et al*., 1998). However, inhibition of this mechanism by corticosteroid hormones associated with stress could increase susceptibility to disease (Miele *et al*., 1998, Simmaco *et al*., 1998).

6.4.2 Carbaryl mediated inhibition of skin peptide defences

The observation that acute exposure of *L. raniformis* to carbaryl induced a decrease in total amount of recovered peptides shows that carbaryl can inhibit skin peptide defences. Since the frogs are more likely to experience multiple exposure in the wild that could have stronger inhibitory effects on skin peptide defences than acute exposures, carbaryl could potentially increase susceptibility to disease.

Decrease in total amount of recovered peptides was not associated with a decrease in peptide activity, nor with any change in the composition of the natural peptide mixtures, based on LC-MS (Figs. 6.3 and 6.4; Table 6.2). This supports the hypothesis of Davidson *et al*. (2007), that carbaryl could decrease peptide deposits in granular glands. This hypothesis was based on the results of experimental studies that showed carbaryl mimics the effects of norepinephrine on short circuit currents (SCC) in skin. In *Rana esculenta*, bathing isolated frog skin in relatively low concentrations of carbaryl induced increased SCC due to secretion of chloride ions (Cl^-), which might be an important stimulus for release of peptides from granular glands (Ardizzone *et al*., 1990, Lippe *et al*., 1992, Erspamer, 1994). Prolonged alterations of SCC could significantly reduce peptide deposits in granular glands. Further histological and chemical studies should a reveal mechanism of effect of carbaryl on reduction of peptide deposits in granular glands.

The significance of inhibition of skin peptide defences by carbaryl for susceptibility to disease of the frogs in the wild might be dependent on a number of different factors. Metamorphs might be more susceptible to effects of carbaryl than adult frogs as the total amount of recovered peptides of *R. boylii* after exposure to carbaryl was decreased five-fold (Davidson *et*

al., 2007) while the total amount of recovered peptides of adult *L. raniformis* after exposure to carbaryl decreased less than 50% (Fig. 6.1; Table 6.2). An important factor that could contribute to susceptibility of skin peptide defences of metamorphs to carbaryl could be the high level of corticosteroids that are associated with metamorphic events (Rollins-Smith, 1998). Some species with less efficient skin peptide defences might be more vulnerable to carbaryl than species with efficient skin peptide defences such as *Xenopus laevis* (described in Chapter 2). In addition, the effects of carbaryl could be enhanced through interactions with environmental conditions such as temperature fluctuations and UV light (Blaustein *et al*. 1998, Belden and Blaustein, 2002, Beecher, 2006). Inhibition of skin peptide defences might be of greater significance for increased susceptibility to virulent pathogens such as chytrid fungi and pathogenic bacteria. These facts prompt directions for futures studies that should focus on how species, age, pathogen virulence and different environmental conditions could affect the carbaryl induced inhibition of skin peptide defences of frogs.

Due to intensive usage of different pesticides and subsequent pesticide drift, frogs could be exposed to different pesticides that could affect skin peptide defences. Gibble *et al* (2006) found that exposure of metamorphs of *X. laevis* to atrazine significantly reduced the efficiency of skin peptide against chytrid fungi. Atrazine could have a different effect on skin peptide defences than carbaryl as it might influence peptide synthesis by increasing the blood level of corticosteroids (Hayes *et al*., 2003, Simmaco *et al*., 1998). Thus, different pesticides could affect different aspects of the skin peptide defence system.

Hayes *et al* (2006) demonstrated that exposure of tadpoles to pesticide mixtures increased susceptibility to disease caused by the bacterium *C. meningosepticum*, while exposure to each

of these pesticides solely did not increase disease development. Tadpoles are likely to have more susceptible immune defence to pesticides than metamorphs and adults due to less developed innate and adaptive immune defences (Flajnik *et al*., 1986, Flajnik *et al*., 1987, Pross and Rowlands, 2005). However, it is plausible that exposure to different pesticides in mixtures might cause both additive and synergetic harmful effects in adults and metamorphs as well, so further research should be directed in this area.

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Chapter 7: Concluding Discussion

The central aims of this study were to determine whether the antimicrobial peptides from skin granular glands of three semi-aquatic *Litoria* frog species *L. aurea, L. raniformis* and *L. ewingii* and aquatic *Xenopus laevis* could have a function against bacterial pathogens associated with bacterial dermatosepticemia, and to investigate whether five different factors: phylogeny, life stage, pathogen, low temperature and pesticides could influence such a function. As disease is caused by different pathogens (Glorioso *et al*., 1974, Anver and Pond, 1984, Olson *et al*., 1992, Taylor *et al*., 2001), the activity of skin peptides was tested against eight different pathogens including: *Aeromonas hydrophila*, *Chryseobacterium meningosepticum*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Serratia liquefaciens*. The single peptides and natural mixture of skin peptides of three species: *L. raniformi*s and *Litoria aurea* and *Xenopus laevis* had activity against more than one pathogen including *Citrobacter freundii*, *Chryseobacterium meningosepticum*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa in vitro*. This could provide significant protection *in vivo.* The presence of effective skin peptides in different frog species indicates they are a conserved mechanism of immune defence. The role of skin peptides in protection from disease is supported by fact that the activity of these skin peptides correlates well with resistance to disease. *L. ewingii*, whose skin peptides did not have activity against any bacterial pathogen, was susceptible to disease both in the wild (Schadich unpubl. data) and following experimental exposure to the pathogen *K. pneumoniae*, while *L. raniformi*s was resistant to that pathogen. The protection from disease provided by skin peptides,

however, could be context-dependent, since it was found that all five tested factors strongly influence their effectiveness.

The immune function against bacterial pathogens of the aquatic species *X. laevis* might be more efficient than those of three tested semi-aquatic *Litoria* species in terms of production and activity of skin peptides. *X. laevis* produced a significantly greater amount of skin peptides than *L. aurea* and *L. raniformis* while *L. ewingii* did not have active peptides. Similarly, the peptide mixture of *X. laevis* had greater activity against bacterial pathogens than all three tested *Litoria* species. The greater activity of peptide mixture correlated with its greater diversity, as observed by LC-MS analyses. The significance of peptide diversity was also supported by the observation that the peptide mixture of *L. ewingii*, which was not active against any tested pathogen, had the smallest peptide diversity.

Three pathogens: *A. hydrophila*, *P. mirabilis* and *S. liquefaciens* showed resistance to skin peptides tested. For one pathogen, *A. hydrophila*, it was demonstrated that resistance to skin peptides was associated with extracellular proteases. Since all three resistant bacterial species were also a component of the microbiota of skin of healthy frogs (Hird *et al*., 1981, Taylor *et al*., 1993, Taylor *et al*., 2001), resistance to such peptides might have a role in their relationship with their hosts. The regulation of these pathogens could include defence mechanisms other than skin peptides such as microbial antagonism and macrophages of skin and lymphatic sinuses.

The analyses of skin peptides of *L. ewingii* as adults, metamorphs and larvae showed that postmetamorphic animals, adults and metamorphs secrete a peptide mixture with a species-

specific profile that includes the antimicrobial peptide uperin 7.1, while the larval peptide mixture did not contain uperin 7.1 or any other known species-specific peptide. These differences in skin peptides between adults, metamorphs and larvae indicates the absence of a secretory mechanism that could compensate for the absence of granular glands in larvae. Since the onset of skin secretions in metamorphosis is a conserved physiological process (Delfino, 1991, Erspamer, 1994, Viertel and Richter, 1999), the differences in skin peptides among different life stages of *L. ewingii* are relevant when considering activity of skin peptides in the different life stages of other frogs species.

Both low temperature and the pesticide carbaryl were shown to have the ability to modulate the function of skin peptides of *L. raniformis*. The endpoint of their effect is in lowering the amount of bio-active skin peptides induced on the skin surface, which can significantly suppress the immune activity against invading pathogens. The intensity of carbaryl-induced suppression of skin peptides is sufficiently strong to disrupt the conserved response mechanism of frogs to increase the amount of induced peptides in response to *K. pneumoniae* challenge. As the skin peptides are the only effective defence of frogs against the frog specific pathogen *Batrachochytrium dendrobatidis* (Rollins-Smith et al. 2002, Woodhams *et al*., 2006, Woodhams *et al*., 2007), a reduced level of skin peptides could substantially impair the immune response against this pathogen. Therefore, the results of this study, showed that both low temperature and pesticide could suppress the innate immune defences of frogs in a manner that could potentially increase susceptibility both to general pathogens such as opportunistic bacteria and to frog-specific pathogens.

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Appendix A: LC-MS/MS Analyses of Peptides of *X. laevis*

Table A. LC-MS/MS analyses of peptides of *X. laevis*.

Distinct peptide	No.	AA	Peptide fragment
CPF 1	gi|115430	27	1-10/GFGSFLGKAL 11-27/KAALKIGANALGGSPQQ
CPF 2	gi|115435	27	1-10/GFASFLGKAL 11-27/KAALKIGANMLGGTPQQ
CPF 3	gi|115432	27	1-10/GLASLLGKAL 3-14/ASLLGKALKAGL 15-27/KIGTHFLGGAPQQ
CPF 4	gi|115434	27	1-10/GLASFLGKAL 3-14/ASFLGKALKAGL 11-27/KAGLKIGAHLLGGAPQQ 15-27/KIGAHLLGGAPQQ
PGL	gi|65026	21	1-18/GMASKAGAIAGKIAKVAL 5-21/KAGAIAGKIAKVALKAL + Amide (C-term) 6-21/AGAIAGKIAKVALKAL.+ Amide (C-term) 12-21/KIAKVALKAL + Amide (C-term)
LPF	gi|126222	25	1-11/GWASKIGQTLG 12-25/KIAKVGLQGLMQPK
Magainin I	gi|126683	23	1-10/GIGKFLHSAG 11-23/KFGKAFVGEIMKS 14-23/KAFVGEIMKS
Magainin II	gi|126683	23	1-7/GIGKFLH 1-10/GIGKFLHSAK 11-23/KFGKAFVGEIMN 14-23/KAFVGEIMNS
PGQ	gi|236997	24	1-11GVLSNVIGYLK

Footnote: No. is the accession number of the peptide sequence in the NCBI database. AA is the total number of amino acids of the distinct peptide. Position of peptide fragments in distinct peptides are denoted by /.

Appendix B: LC-MS/MS Analyses of Peptides of *L. aurea*

Table B. LC-MS/MS analyses of peptides of *L. aurea*.

Aurein	No.	AA	Peptide fragment
2.2	gi|20137309	16	1-8/GLFDIVKK
			1-11/GLFDIVKKVVG
			3-11/FDIVKKVVG
			3-16/FDIVKKVVGALGSL+ Amide (C-term)
2.3	gi|20137310	16	1-8/GLFDIVKK
			1-11/GLFDIVKKVVG
			3-16/FDIVKKVVGAIGSL+ Amide (C-term)
2.4	gi|20137311	16	1-8/GLFDIVKK
			1-9/GLFDIVKKV
			1-11/GLFDIVKKVVG
			3-11/FDIVKKVVG
			3-16/FDIVKKVVGTIAGL+ Amide (C-term)
2.5	gi|68053337	16	1-8/GLFDIVKK
			1-11/GLFDIVKKVVG
			3-11/FDIVKKVVG
			3-16/FDIVKKVVGAFGSL+ Amide (C-term)
3.1	gi|68053340	17	1-8/GLFDIVKK
			3-17/FDIVKKIAGHIAGSI+ Amide (C-term)
3.2	gi|68053342	17	1-7/GLFDIVK
			1-8/GLFDIVKK
			3-8/FDIVKK
			1-9/GLFDIVKKI
			1-11/GLFDIVKKIAG
			3-11/FDIVKKIAG
			1-12/GLFDIVKKIAGH
			1-14/GLFDIVKKIAGHIA
			1-16/GLFDIVKKIAGHIASS
5.2	gi|57975125	25	1-11/GLMSSIGKALG
			5-24/SIGKALGGLIVDVLKPKTPA
			8-24/KALGGLIVDVLKPKTPA
			12-24/GLIVDVLKPKTPA
			12-25/GLIVDVLKPKTPAS

Footnote: No. is the sequence number of the aurein peptides sequences in the NCBI database. AA is the total number of amino acids of the distinct aureins. Position of peptide fragments in peptide sequence are denoted by /.

Appendix C: LC-MS/MS Analyses of Peptides of *L. raniformis*

Table C. LC-MS/MS analyses of peptides of *L. raniformis*. Table agenda as in Table B (Appendix B)

Aurein	No.	AA	Peptide fragment
1.2	gi|20137307	13	1-11/GLFDIIKKIAE
			3-13/FDIIKKIAESF+ Amide (C-term
			4-14/DIIKKIAESF+ Amide (C-term)
2.1	gi|68053336	16	1-11/GLLDIVKKVVG
			2-16/LDIVKKVVGAFGSL+ Amide (C-term)
2.6	gi|20137313	16	1-11/GLFDIAKKVIG
3.1	gi|68053340	17	1-8/GLFDIVKK
			3-17/FDIVKKIAGHIAGSI+ Amide (C-term)
3.2	gi|68053342	17	1-7/GLFDIVK
			1-8/GLFDIVKK
			3-8/FDIVKK
			1-9/GLFDIVKKI
			1-11/GLFDIVKKIAG
			3-11/FDIVKKIAG
			1-12/GLFDIVKKIAGH
			1-14/GLFDIVKKIAGHIA
3.3	gi|20137316	17	1-7/GLFDIVK
			1-8/GLFDIVKK
			3-11/FDIVKKIAG
			1-11/GLFDIVKKIAG
5.2	gi|57975125	25	1-11/GLMSSIGKALG
			5-24/SIGKALGGLIVDVLKPKTPA
			8-24/KALGGLIVDVLKPKTPA
			12-24/GLIVDVLKPKTPA
			12-25/G.GLIVDVLKPKTPAS

Footnote is as in Table B (Appendix B).

Appendix D: LC-MS/MS Analyses of Peptides of *L. ewingii*

Table D. LC-MS/MS analyses of peptides of *L. ewingii*. Table agenda as in Table A (Appendix A)

Distinct peptide	No.	AA	Peptide fragment
Uperin-7.1	gi|7994724	13	1-7/GWFDVVK
			1-8/GWFDVVKH
			3-10/FDVVKHIA
			3-13/FDVVKHIASAV + Amide (C-term)
			4-13/DVVKHIASAV + Amide (C-term)

Footnote is as in Table A (Appendix A).

Appendix E: List of Peptide Masses in Peptide Digest of *X. laevis*

Table E. List of molecular masses of peptide digests of *X. laevis*. Peaks of LC-MS analyses are labelled by numbers.

Peak	M_r (Da)
1	821.76
2	801.10 996.90 1208.76
3	801.13 1467.18
4	330.8
5	557.77 972.59 1431.26
6	487.799 905.61 972.59 1431.59
7	369.18 387.91 445.77 626.77 1160.73

Appendix F: List of Peptide Masses in Peptide Digest of *L. aurea*

Table F. List of Molecular masses of peptide digests *L. aurea*. Table agenda as in Table E (Appendix E).

Peak	M_r (Da)
1	703.29 899.06 1094.87
2	784.20 899.06
3	330.90 390.95
4	245.92 376.92 390.96
5	447.84 1022.85

Appendix G: List of Peptide Masses in Peptide Digest of *L. raniformis*

Table G. List of Molecular masses of peptide digests *L. raniformis*. Table agenda as in Table E (Appendix E).

Peak	M_r (Da)
1	594.94 742.02
2	703.31
3	524.94 785.92
4	343.88 444.87
5	488.81 1127.72
6	975.02
7	341.96 383.94 456.88 739.84
8	447.64 564.90 1039.58

Appendix H: List of Peptide Masses in Peptide Digest of *L. ewingii*

Table H. List of Molecular masses of peptide digests *L. ewingii.* Table agenda as in Table E (Appendix E).

Peak	M_r (Da)
1	508.48 605.35 686.21 1192.70
2	204.95 443.80 507.50
3	255.95 390.95
4	261.90 431.87 487.83 519.89 603.76
5	272.92 413.92 457.95 460.02 478.85 1171.78

Appendix I: List of Peptide Masses in Peptide Digest of Metamorphs of *L. ewingii*

Table F. List of Molecular masses of peptide digests of metamorphs (A) and adults (B) of *L. ewingii.* Table agenda as in Table E (Appendix E).

A.

Peak	M_r (Da)
1	446.42 811.30
2	909.34 593.18
3	909.34
4	1848.78

B.

Peak	M_r (Da)
1	508.48 605.32 204.95
2	204.95 443.80 507.50
3	431.87
4	431.87 603.76
5	478.85 117.78

Appendix J: Calibration Curves for Bacterial Counts

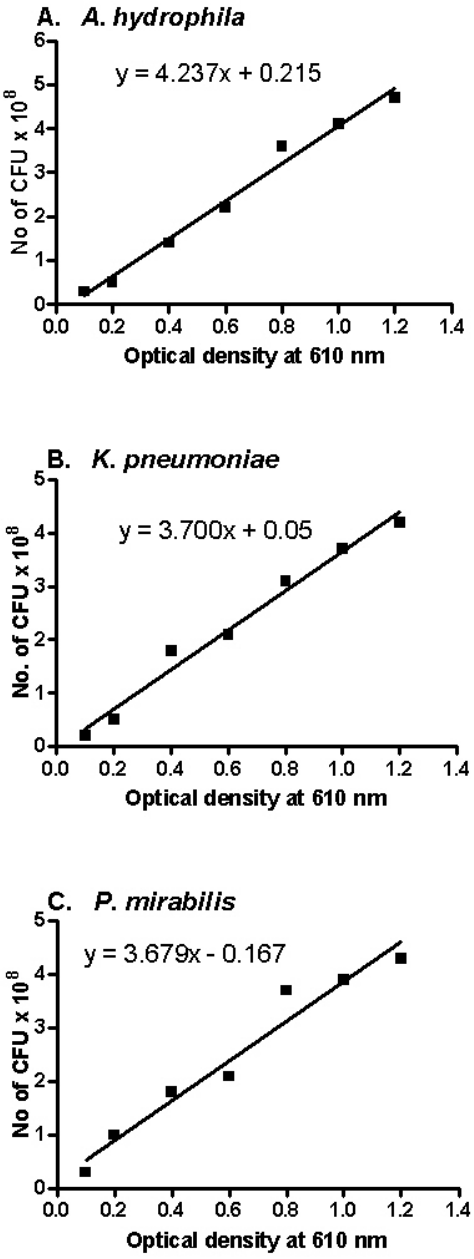

Fig. F Calibration curves for counting colony forming units of *A. hydrophila* (A), *K. pneumoniae* (B) and *P. mirabilis* (C).

Appendix K: Assessment of Bacterial Motility

Bacterial inoculum was stabbed in the centre and to 2/3 the depth of API M medium (Biomérieux, Marcy-L'etoile, France). The samples were incubated for 18 h at 30ºC and bacterial growth was examined visually. Diffuse circular bacterial growth from the line of stab shows positive test.

Appendix L: Bacterial Growth on MacConkey Agar

Bacterial suspension was spread onto plates of MacConkey agar and incubated for 18 h at 30ºC. After incubation the growth was examined visually.

Appendix M: Glucose Oxidation/ Fermentation (O/F) test

Glucose oxidation fermentation test (O/F) test demonstrates the ability of bacteria to metabolise glucose aerobically (by oxidation) or anaerobically (by fermentation). Tests was performed using O-F basal medium (Difco, Lawrence, KS, USA) with 1% glucose. A loopful of bacterial suspension was inoculated into ten ml of medium in two test tubes. Sterile liquid paraffin was added to a depth of 1 cm in one tube while the other was left uncovered. Tubes were incubated overnight at 30ºC. Results were interpreted as follows:

Test	Open tube	Covered
Oxidation	Yellow	Green
Fermentation	Yellow	Yellow
No reaction	Blue/Green	Green

Appendix N: Biochemical Profiles of Bacteria Isolated from Diseased *L. ewingii* in *K. pneumoniae* Challenge

Table 5.2. Results of API 20 E strip tests and four supplementary tests for bacteria isolated from diseased *L. ewingii* in *K. pneumoniae* challenge.

Test	Bacteria from infected tissues (N = 2 animals)			Challenge Bacteria
	Heart	Liver	Spleen	
β-galactosidase	+	+	+	+
Arginine dihydrolase	–	–	–	–
Lysine decarboxylase	+	+	+	+
Ornithine decarboxylase	–	–	–	–
Citrate utilisation	+	+	+	+
H_2S production	–	–	–	–
Urea	+	+	+	+
Tryptophane deaminase	–	–	–	–
Indole production	–	–	–	–
Voges-Proskauer	+	+	+	+
Gelatin hydrolysis	–	–	–	–
D-glucose	+	+	+	+
D-mannitol	+	+	+	+
Inositol	+	+	+	+
D-sorbitol	+	+	+	+
L-rhamnose	+	+	+	+
D-surcose	+	+	+	+
D-melibiose	+	+	+	+
amygdalin	+	+	+	+
L-arabinose	+	+	+	+
Cytochrome oxidase	–	–	–	–
NO_2 production	+	+	+	+
Reduction to N_2	–	–	–	–
Motility*	–	–	–	–
Growth on MacConkey medium*	+	+	+	+
Fermentation on glucose*	+	+	+	+
Oxidation of glucose*	+	+	+	+

Footnote: * denotes supplementary test.